ATTACHMENT A

K. Papp, Arch. Dermatol. Res., Vol. 298, pages 7-15 (2006).

J. Arthur et al., Journal of Investigative Dermatology, Vol. 126, pages 1689-1691 (2006).

A. Gottlieb et al., The Journal of Immunology, Vol. 175, pages 2721-2729 (2005).

C. Johansen et al., The Journal of Immunology, Vol. 176, pages 1431-1438 (2006).

A. Funding et al., Journal of Investigative Dermatology Advance Online Publication, doi:10.1038/sj.jid.5700252 (March 16, 2006).

C. Johansen et al., British Journal of Dermatology, Vol. 152, pages 37-42 (2005).

X. Yu et al., Experimental and Molecular Pathology, Vol. 83, pages 413-418 (2007).

M. Lowes et al., Proceedings in the National Academy of Sciences, Vol. 102, No. 52, pages 19057-19062 (2005).

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MINI-REVIEW

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The long-term efficacy and safety of new biological therapies for psoriasis

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Abstract Long-term therapy is often required for psoriasis. This article reviews the most recent long-term clinical data for biological agents that have been approved or for which late-stage development data have been released for the treatment of patients with moderate to severe plaque psoriasis. Efficacy data are available for up to five 12-week courses of alefacept (approximately 60 weeks of therapy), 36 months (144 weeks) of continuous efalizumab, 48 weeks of continuous etanercept, and 50 weeks of bimonthly infliximab. Data sources include publications, product labeling, and posters presented at recent international scientific meetings. Alefacept appears to continue to be efficacious over multiple treatment courses for some responsive patients. The efficacy of efalizumab achieved during the first 12–24 weeks of therapy appears to be maintained or improved through at least 60 weeks of continuous treatment. The efficacy of etanercept appears to be maintained through at least 48 weeks of continuous treatment. Infliximab demonstrates a high response rate soon after initiation, which appears to be maintained through 24 weeks but declines modestly with therapy out to 50 weeks. After 48 weeks, approximately 60% of efalizumab-treated and 45% of etanercept-treated patients remaining on therapy achieved ≥75% improvement from baseline in Psoriasis Area and Severity Index, as did 70.5% of infliximab patients who did not miss more than two infusions. Safety data suggest that these agents may be used for long-term administration. Long-term data from psoriasis trials continue to accumulate. Recent data suggest that biological therapies have efficacy and safety profiles suitable for the long-term treatment of patients with moderate to severe psoriasis.

Keywords Biological · Alefacept · Efalizumab · Etanercept · Infliximab · Long-term treatment · Psoriasis

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Introduction

Psoriasis is a chronic inflammatory, immune-mediated disease characterized by periods of spontaneous remission and relapse [22]. Conventional nonbiological therapies for moderate to severe psoriasis, such as cyclosporine, methotrexate, and phototherapy, have proved effective in suppressing symptoms; and many dermatologists have at least a decade of experience using these treatment modalities [21]. Because of the chronic nature of psoriasis, many patients require lifelong symptom management. Conventional nonbiological systemic therapies have been successfully used as continuous therapy in some patients for extended periods (e.g., methotrexate); however, for some patients, the long-term use of these agents may be limited by known toxicities, including nephrotoxicity (cyclosporine) [21], hepatotoxicity (methotrexate) [10], skin cancer (psoralen-ultraviolet A phototherapy, or PUVA) [23], and prolonged teratogenicity (oral retinoids) [5].

Current treatment guidelines recommend limiting the administration of cyclosporine to short-term (3-month) intermittent therapy or to no longer than 1–2 years continuously [11], reducing the dosage and minimizing lifetime exposure to methotrexate and ultraviolet irradiation [12, 27], and avoiding treatment with oral retinoids and several other conventional antipsoriatic therapies in women of childbearing potential [4]. Available data suggest that, when used according to contemporary guidelines and with appropriate monitoring for toxicity, conventional nonbiological treatments can be reasonably safe and tolerable [29]. However, minimizing toxicity while still providing continuous control

of psoriasis symptoms over a patient's lifetime represents a clinical challenge.

An improved understanding of the immunologic basis of psoriasis led to the development of several targeted biological therapies that attempt to address the challenge of providing long-term continuous disease control. The first biological agent approved for the treatment of adult patients with moderate to severe chronic plaque psoriasis, alefacept (Amevive®, Biogen Idec Inc.), is currently registered for use in several countries, including the United States but not the European Union (EU). Two others, efalizumab (Raptiva®, Genentech, Inc.; Serono International S.A.) and etanercept (Enbrel®, Amgen, Inc.; Wyeth Pharmaceuticals), are also registered for this indication in multiple countries, including the EU and the United States. Another biological, infliximab (Remicade®, Centocor, Inc.; Schering-Plough Corp.), has presented Phase III clinical trial data for the treatment of psoriasis and recently became registered for use in the EU. The clinical trials programs for these four agents have demonstrated their safety and efficacy in patients with psoriasis over an initial period of several months; longer-term results are now becoming available. One other biological agent, adalimumab (Humira®, Abbott Laboratories), is at an earlier stage of development for psoriasis and has completed Phase II clinical trials for the disease. This article reviews the latest clinical data available regarding the long-term efficacy of these biological agents for treatment of psoriasis.

Alefacept

Alefacept is a fusion protein between human leukocyte function-associated antigen-3 (LFA-3) and the human IgG_1 antibody Fc region. By binding to the CD2 antigen on T cells, alefacept prevents T-cell activation and triggers apoptosis, preferentially targeting memory-effector T cells [3]. Alefacept has been evaluated as a weekly intravenous (IV) 7.5-mg administration and as a weekly intramuscular (IM) 15-mg injection (Amevive® [alefacept] prescribing information; Cambridge, Mass: Biogen, Inc.; August 2004); however, only the IM dose is currently available. The efficacy and safety of a 12-week course of weekly IM alefacept 15 mg were evaluated in a randomized, placebo-controlled Phase III trial of adult patients with chronic plaque psoriasis [minimum of 10% body surface area (BSA) affected by psoriasis] [15]. Two weeks after the treatment phase was completed (study week 14), the Psoriasis Area and Severity Index (PASI) improved by at least 75% from baseline (PASI-75) in 21% of the 166 patients who received alefacept 15 mg per week, with 42% achieving at least a 50% improvement from baseline (PASI-50) (Amevive® [alefacept] prescribing information). This compares with rates of 5 and 18%, respectively, for patients randomized to placebo ($n=168$; $P<0.001$ for both comparisons). Mean percentage PASI improvement from baseline for a course of alefacept 15-mg IM therapy is shown in Fig. 1.

Alefacept has demonstrated the longest psoriasis remission times among biological agents for psoriasis. Patients who achieved PASI-75 after a 12-week course of alefacept 15 mg per week ($n=54$) maintained at least a PASI-50 response for a median period of approximately 7 months [15]. Thus, the long-term use of alefacept as a remittive therapy has been explored through extension studies of additional treatment courses for patients who were enrolled in alefacept IV or IM Phase III trials. Efficacy data have been presented for up to five 12-week courses of alefacept therapy (i.e., approximately 60 weeks of treatment), although PASI responses are available for only the IV formulation [IM results were provided using Physician's Global Assessment (PGA)] (A. Menter et al., unpublished data presented at the 63rd Annual Meeting of the American Academy of Dermatology, 2005). The analyses were performed on the as-treated patient population, and a patient was considered a responder if he or she achieved a response anytime during each 12-week treatment course. Using these criteria, PASI-75 response rates for IV alefacept increased from 29% during the first course of therapy ($n=521$) to 54% during course 5 ($n=39$). PGA response rates for the IM formulation increased from 21% of patients classified as "clear" or "almost clear" during course 1 ($n=457$) to 41% during course 4 ($n=100$), but the response rate decreased to less than 30% during course 5 ($n=50$).

Alefacept therapy appears to be well tolerated, even with long-term use. The primary concern with alefacept is T-lymphocyte depletion. Alefacept should not be initiated in patients with $CD4^+$ T-lymphocyte counts below normal, and biweekly $CD4^+$ T-cell monitoring is recommended during each treatment course (Amevive® [alefacept] prescribing information). Alefacept should be withheld if T-cell counts fall below 250 cells/μl and discontinued if they remain low for a month. Patients treated with alefacept during Phase III trials demonstrated a low incidence of malignancy, serious infections requiring hospitalization, and hypersensitivity reactions (Amevive® [alefacept] prescribing information). The integrated safety database for alefacept includes a total of 1,869 alefacept-treated psoriasis patients and includes patients who have received up to nine courses of alefacept therapy ($n=8$) over a 5-year period. Despite the paucity of data for multiple treatment courses, the incidence of common adverse events (e.g., headache, nasopharyngitis, influenza, upper respiratory tract infection, and pruritus), serious adverse events, infections, malignancies, and autoantibody production appeared to fluctuate little with additional treatment, although the safety of long-term alefacept therapy will need to continue to be assessed.

Efalizumab

Efalizumab is a recombinant humanized monoclonal IgG_1 antibody against the α subunit (CD11a) of LFA-1,

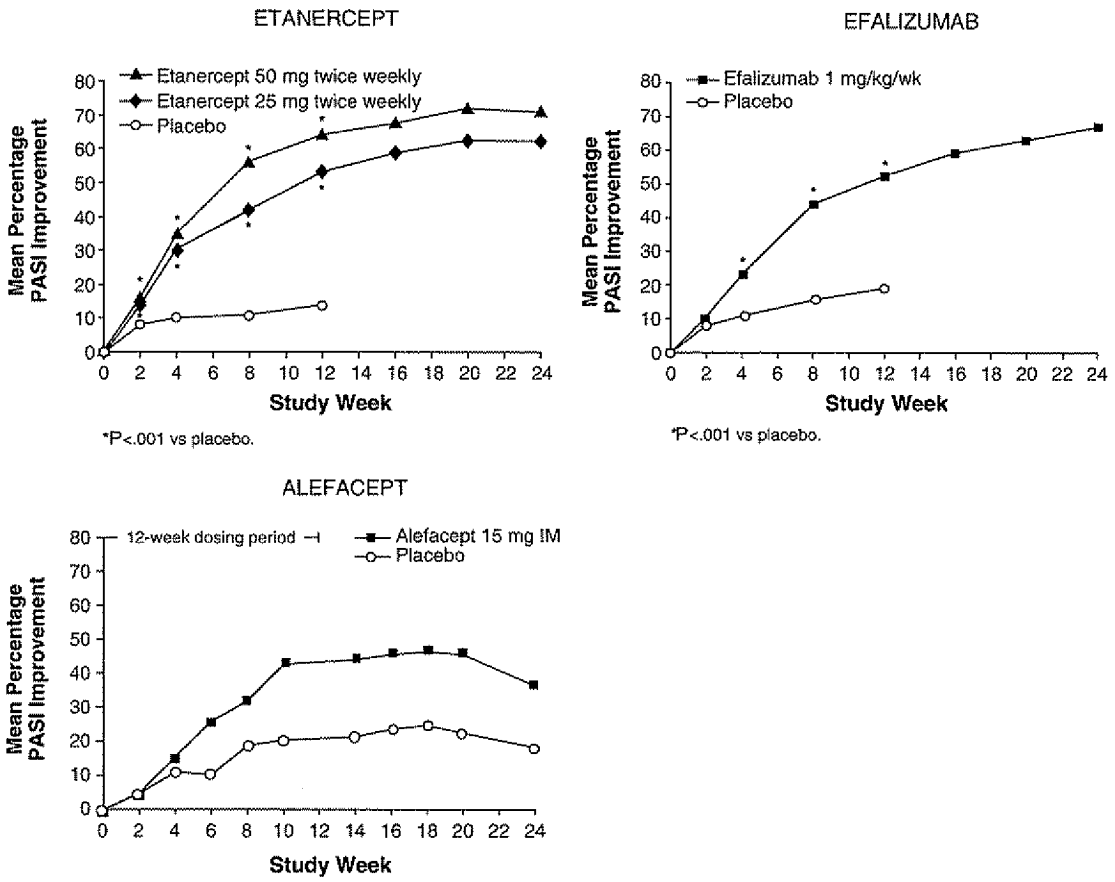

Fig. 1 Mean percentage psoriasis area and severity index (*PASI*) improvement from baseline in randomized placebo-controlled, Phase III studies of biological agents for the treatment of psoriasis. *ETANERCEPT* Results for etanercept 25 or 50 mg twice weekly for 24 weeks [18]. Sample size at each PASI assessment was not reported. *EFALIZUMAB* Results for the use of efalizumab 1 mg/kg/week for 12 weeks followed by a 12-week open-label extension phase [6, 19]. The efficacy-evaluable population for this study included all patients randomized to efalizumab treatment ($n=369$), which differs from other efalizumab trials (and those of the other agents discussed) that include only patients who received at least 1 dose of the study drug. Following are the sample sizes at each PASI assessment, represented by the data points. For efalizumab: *week 0*, 369; *week 2*, 357; *week 4*, 353; *week 8*, 352; *week 12*, 347; *week 16*, 322; *week 20*, 323; *week 24*, 312. For placebo: *week 0*, 187; *week 2*, 186; *week 4*, 184; *week 8*, 180; *week 12*, 175 [19]. *ALEFACEPT* Alefacept results for the available 15-mg intramuscular (*IM*) dose [15]. Drug was administered weekly for 12 weeks, followed by observation for 12 weeks. In this study, 166 patients were randomized to receive alefacept 15 mg IM and 168 patients to receive placebo. Sample sizes at each PASI assessment were not provided. Statistical comparisons were not reported. *Note* Data for infliximab were not available

which modulates several key steps in the immunopathogenesis of psoriasis, including initial T cell activation, trafficking from the circulation into psoriatic lesions, and reactivation therein [13]. Multiple Phase III clinical trials have demonstrated the efficacy, safety, and health-related quality-of-life (HRQOL) benefits of 12 weeks of subcutaneous (SC) efalizumab therapy in patients with moderate to severe chronic plaque psoriasis [6, 16, 20, 24, 28]. As will be discussed later, available clinical data support the efficacy of extending efalizumab treatment beyond 12 weeks, as reflected in the European guidelines (European Medicines Agency: Raptiva [efalizumab] summary of product characteristics; http://www.emea.eu.int/humandocs/Humans/EPAR/Raptiva/raptiv/htm; 2005).

The Phase III study that served as the basis for efalizumab labeling evaluated the efficacy and safety of efalizumab 1 mg/kg weekly [6]. A total of 556 adult patients with moderate to severe chronic plaque psoriasis (baseline PASI ≥12.0 and ≥10% BSA affected) were randomized to receive efalizumab ($n=369$) or placebo ($n=187$) double-blind for 12 weeks; all patients were then eligible to receive extended efalizumab open-label treatment for an additional 12 weeks [19]. A significant efalizumab treatment effect relative to placebo was achieved at 12 weeks, and extended treatment conferred additional clinical benefit. Using an intent-to-treat (ITT) analysis with the last observation carried forward (LOCF) for patients with missing data, the percentage of efalizumab-treated patients who achieved PASI-75 increased from 27% at week 12 (vs. 4% with placebo, $P<0.001$) to 44% at week 24. Similarly, the percentage of efalizumab-treated patients who achieved PASI-50 and PASI-90 increased from 59 to 67% and from 5 to

15% at weeks 12 and 24, respectively. The mean percentage PASI improvement relative to baseline increased from 52% at week 12 to 67% at week 24 (Fig. 1) [6, 19]. Mean percentage PASI improvement appeared to increase throughout the 24-week duration without any evidence of a plateau effect. Two additional Phase III studies with similar placebo-controlled, double-blind initial 12-week treatment periods support the efficacy and safety of efalizumab therapy over 24 weeks [16, 17].

An open-label Phase III study evaluated the efficacy, safety, and tolerability of up to 36 months of continuous efalizumab treatment. At the time of this writing, results through 60 weeks of the trial and through 27 months (108 weeks) of the trial have been published [7, 9] and data through 36 months (144 weeks) have been presented (A.B. Gottlieb et al., unpublished results reported at the 63rd annual meeting of the American Academy of Dermatology, 2005). A total of 339 patients with moderate to severe plaque psoriasis received weekly efalizumab 2 mg/kg for 12 weeks; of the 308 patients who completed the initial treatment period, 290 patients (94%) achieved PASI-50 or a static Physician's Global Assessment (sPGA) of mild, minimal, or clear at week 12 and were allowed to receive maintenance treatment with efalizumab 1 mg/kg per week for up to 33 additional months (132 weeks) [7, 9]. At completion of the first 12-week treatment period, 41% of patients achieved PASI-75. Preliminary results showed that, by week 60, PASI-75 responses were observed in 49% of the ITT population ($n=339$) and in 57% of patients ($n=290$, last observation carried forward) who were analyzed by means of a maintenance group analysis, which included only patients eligible to receive continued efalizumab. An as-treated analysis indicated that 64% of patients ($n=228$) achieved PASI-75 at week 60 (PASI-75 results through week 48 are shown in Fig. 2) [7]. Results in this trial support the sustained efficacy of up to 36 months of continuous efalizumab treatment (A.B. Gottlieb et al., unpublished results reported at the 63rd annual meeting of the American Academy of Dermatology, 2005).

Some limitations of the 3-year study are noted. At initiation of the trial, the label-supported dosage of 1 mg/kg had not been established; thus, a dosage of 2 mg/kg was administered during the initial 3-month period. It was subsequently demonstrated that the safety and efficacy do not differ for the 1- and 2-mg/kg doses [16, 17]. In addition, although relatively few patients were affected ($\leq 4\%$), a dose increase of up to 4 mg/kg was allowed up through 15 months of the study. Use of the LOCF procedure during analysis of ITT data might introduce bias in the final result; depending on the response of the patient, the bias could overestimate or underestimate the final result.

Clinical trials have shown that efalizumab is generally well tolerated and has a favorable safety profile over an initial 12-week treatment period [6, 16, 24, 28]. Results of the extended studies described here support the continued safety and tolerability of efalizumab treatment periods longer than 12 weeks [7, 9, 16, 17, 19]. The most common adverse events associated with efalizumab administration are acute flulike symptoms (headache, chills, fever, myalgia, vomiting, and nausea) observed primarily following the first two doses. After the third and subsequent doses, the incidence of acute adverse events in efalizumab-treated patients is comparable to that observed in placebo recipients. The extended treatment studies have shown that, with the exception of an expected reduction in acute adverse events, the incidence and intensity of adverse events during the second and subsequent treatment periods are similar to those observed during the first 12 weeks. To date, no evidence of cumulative or end-organ toxicity has been found with efalizumab. Thrombocytopenia has been reported during efalizumab clinical trials (Raptiva® [efalizumab] package insert; South San Francisco, Calif: Genentech, Inc.; June 2005), and recently a statement about the development of hemolytic anemia has been added to the prescribing information (Raptiva® [efalizumab] package insert). Worsening of psoriasis and psoriasis variants has been observed in 3% of efalizumab patients during therapy (i.e., generalized inflammatory flare), and in 14% of patients following abrupt discontinuation of efalizumab (i.e., rebound) [1]. The likelihood of experiencing rebound was inversely related to the PASI response at week 12; 72% of patients experiencing rebound were nonresponders, and only 10% had achieved PASI-75 [1]. New-onset or worsening arthritis has been infrequently reported during clinical trials and postmarketing (Raptiva® [efalizumab] package insert). Based on available clinical data from up to 36 months of continuous treatment, efalizumab does not appear to be associated with an increased risk of malignancy or opportunistic infections observed in immunosuppressed hosts [7, 16, 17, 19, 28]. Because the numbers of patients evaluated in the long-term studies have been relatively small, the incidence of malignancy and infection will be assessed postmarketing (Raptiva® [efalizumab] package insert).

Etanercept

Etanercept is a recombinant fusion protein comprising domains of the 75-kDa human tumor necrosis factor (TNF) receptor and human IgG_1, which inhibits the activity of TNF-α, a proinflammatory cytokine implicated in psoriasis. The efficacy, safety, and HRQOL benefits of 12 and 24 weeks of etanercept therapy in patients with moderate to severe chronic plaque psoriasis (baseline PASI ≥ 10 and $\geq 10\%$ of BSA affected) have been demonstrated in Phase III clinical trials [18, 25]. There is also clinical experience with etanercept in other indications, such as rheumatoid arthritis (RA). Recommended dosage in the EU is 25 mg administered SC twice weekly for up to 24 weeks. Dosing at 50 mg twice weekly is also possible for the first 12 weeks followed by

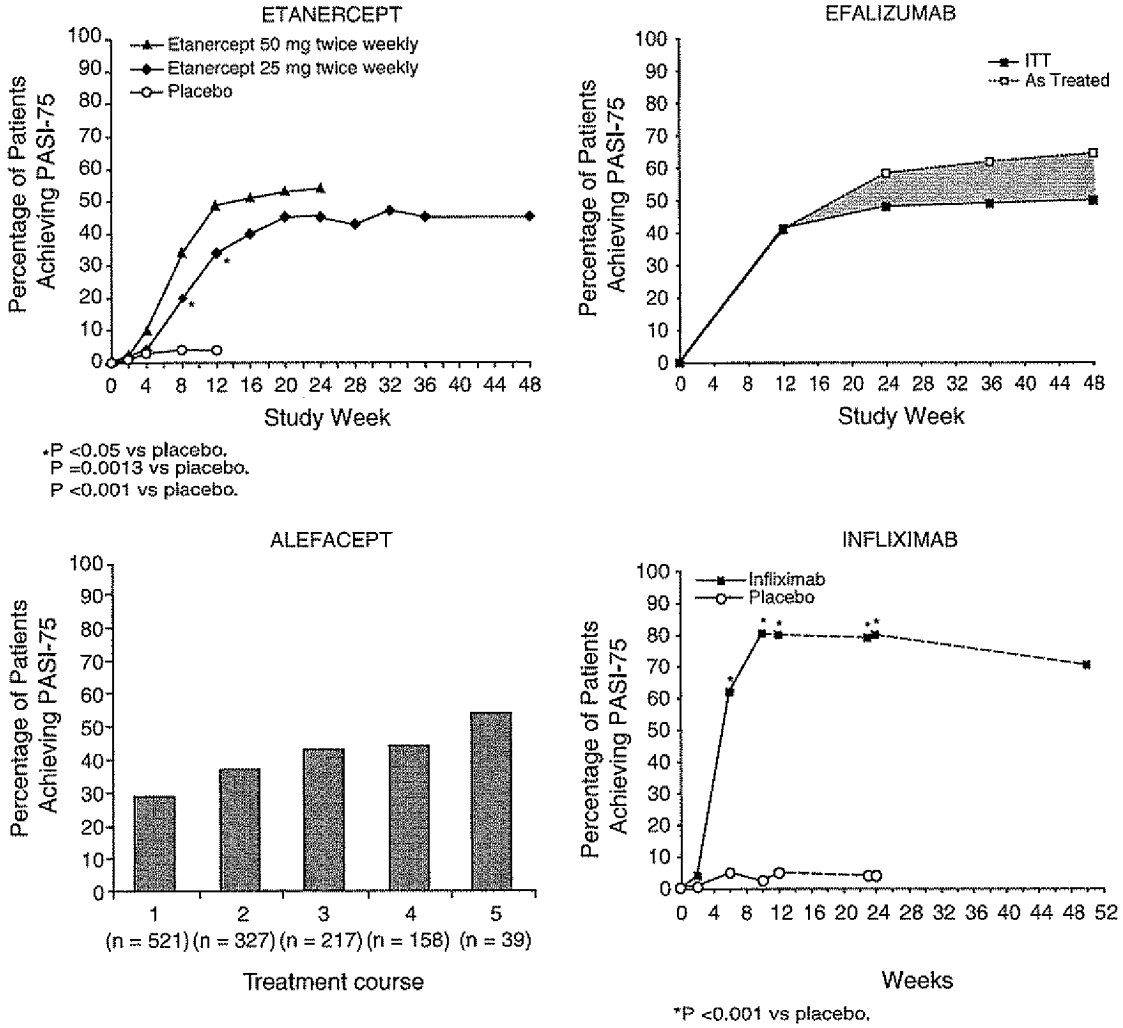

Fig. 2 Percentage of patients achieving 75% improvement from baseline psoriasis area and severity index (*PASI*). *Solid lines* indicate intention-to-treat analyses; *broken lines* indicate as-treated analyses (efalizumab) or modified ITT analysis (infliximab) *ETANERCEPT* Results shown are from an etanercept study in which patients received etanercept 25 mg ($n=196$), etanercept 50 mg ($n=194$), or placebo ($n=193$) twice weekly for 12 weeks followed by open-label etanercept 25 mg twice weekly for up to 36 additional weeks [25]. Efficacy was evaluated in the ITT population. Published data beyond 24 weeks are not available; however, the data presented at several international medical congresses [e.g., European Academy of Dermatology and Venereology (*EADV*), American Academy of Dermatology (*AAD*)] suggest that the additional efficacy initially achieved with etanercept 50 mg twice weekly beyond that obtained with the 50-mg-per-week dose might not be sustained in the long term following dose reduction (unpublished data presented at the 2004 EADV and 2005 summer AAD meetings). *EFALIZUMAB* Results are shown for efalizumab from an open-label Phase III study ($n=339$) of efalizumab 2 mg/kg/week for 12 weeks, followed by 1 mg/kg/week for up to 33 additional months in patients who achieved PASI-50 or a static Physician's Global Assessment (*sPGA*) of mild, minimal, or clear at week 12 [7]. The dosage received during the first 12 weeks was higher than that later approved for clinical use (1 mg/kg/week). Efficacy was evaluated in the intent-to-treat (*ITT*) population throughout the study and is presented through 48 weeks; as-treated analysis is shown after 12 weeks through week 48. Sample sizes for as-treated analysis are as follows: *week 24*, 290; *week 36*, 269; *week 48*, 247 [7]. The *shaded portion* specifies a response window framed by the most conservative measure of efficacy (*ITT*) and the response of patients that remain on therapy (as-treated). *ALEFACEPT* Results are shown for alefacept 7.5 mg administered intravenously (*IV*) (A. Menter et al., unpublished data presented at the 63rd annual meeting of the American Academy of Dermatology); long-term PASI-75 response rates for the alefacept intramuscular (*IM*) dosing regimen have not been reported. Each course represents 12 weekly alefacept infusions followed by a minimum of 12 weeks of treatment-free observation. The second course was initiated 12 weeks after the first course [14]; additional treatment courses were initiated when the patient was deemed by the investigator to require systemic therapy or phototherapy for psoriasis recurrence. Course 5 represents patients who may have received up to 60 total weeks of alefacept treatment. Sample sizes are as-treated numbers. *INFLIXIMAB* Results are shown for infliximab patients who were randomized to receive IV infliximab 5 mg/kg or placebo at initiation, week 2, week 6, and then every 8 weeks through week 46 [26]. Analyses through week 10 were performed using the ITT population (infliximab, $n=301$; placebo, $n=77$); analyses through week 50 were based on a "modified ITT" population ($n=234$) where missing data were considered as nonresponse for patients who withdrew from the study due to preselected reasons (mainly related to lack of efficacy; patients who withdrew for other reasons were omitted from the analysis)

a "step-down" to 25 mg twice weekly. Based on published clinical data, European guidelines indicate that nonresponders should discontinue etanercept after 12 weeks and that reinitiation of etanercept treatment is possible after discontinuation (European Medicines Agency. Enbrel [etanercept] summary of product characteristics; http://www.emea.eu.int/humandocs/Humans/EPAR/enbrel/enbrel.htm; 2005). In the United States, a 50-mg prefilled syringe recently became available, and the recommended dosing of etanercept is 50 mg twice weekly for the first 12 weeks of treatment, followed by step-down to 50 mg once weekly (Enbrel® [etanercept] package insert; Thousand Oaks, Calif: Immunex Corporation; October 2005).

A placebo-controlled, double-blind Phase III study demonstrated the safety and efficacy of 12 and 24 weeks of etanercept treatment [18]. A total of 672 patients were randomized 1:1:1:1 to receive placebo or etanercept 25 mg once weekly, 25 mg twice weekly, or 50 mg twice weekly via SC injection for 12 weeks. Etanercept recipients who completed the initial treatment period continued on their assigned dosage for an additional 12 weeks. Whereas efficacy was originally reported only in patients who received at least 1 dose of study drug ($n=652$) [18], some data were subsequently reanalyzed for all patients who were enrolled and randomized, irrespective of whether the study drug was administered (Enbrel® [etanercept] package insert). These data are discussed herein to allow for a more accurate comparison with ITT data from efalizumab clinical trials. This reanalysis showed that among patients randomized to receive etanercept 25 or 50 mg twice weekly (the dosages most similar to those now recommended in Europe and the United States), 32 and 47% of patients, respectively, achieved PASI-75 at week 12, increasing to 41 and 54% at week 24 (Enbrel® [etanercept] package insert). The mean percentage PASI improvement reported in patients who received at least 1 dose of etanercept 25 or 50 mg increased from 53 to 64%, respectively, at week 12 to 62 and 71% at week 24. However, it appears that the mean percentage PASI improvement began to plateau around week 20 (Fig. 1).

Results from the first 24 weeks of a 48-week continuous etanercept therapy global Phase III psoriasis study were recently published. In the initial 12-week, placebo-controlled, double-blind phase of the study, 611 patients were randomized 1:1:1 to receive placebo or etanercept 25 or 50 mg twice weekly; all patients were then eligible to receive open-label etanercept 25 mg twice weekly for up to 36 additional weeks. Results at 12 and 24 weeks were reported for all randomized patients who received at least 1 dose of study drug ($n=583$) using the LOCF procedure for analysis and for all randomized patients regardless of whether they received study drug ($n=611$), with the assumption that patients with missing data had not met the criteria for response at that end point [25]. At 12 weeks, 32 and 46% of all patients randomized to receive etanercept 25 or 50 mg twice weekly, respectively, achieved PASI-75 (vs. 3% for placebo, $P<0.0001$ both comparisons). The PASI-75 response rate was slightly higher among patients who received at least 1 dose of etanercept 25 or 50 mg (34 and 49%, respectively), increasing to 45 and 54% at week 24 [25]. Data presented for this study at scientific meetings [2004 meeting of the European Academy of Dermatology and Venereology [EADV] and 2005 meeting of the American Academy of Dermatology (AAD)] indicated that among patients continuing on etanercept 25 mg twice weekly, the PASI-75 response achieved at week 24 was maintained through week 48 of continuous treatment and suggested that the greater PASI-75 response rate initially observed after 24 weeks in the group initially randomized to etanercept 50 mg twice weekly decreased slightly after per-protocol dose reduction, with PASI-75 response rates reaching a similar level in both groups during the open-label treatment phase (W. Sterry, unpublished data).

A randomized, placebo-controlled, multicenter Phase III study to evaluate the safety and efficacy of etanercept 50 mg twice weekly over the course of 2 years is currently ongoing. Preliminary results after 48 weeks of therapy were recently reported (S.K. Tyring et al., unpublished data presented at the summer meeting of the American Academy of Dermatology, 2005). In this trial, patients were randomized to receive either etanercept 50 mg ($n=311$) or placebo twice weekly for 12 weeks ($n=307$), followed by open-label etanercept 50 mg twice weekly. Etanercept demonstrated statistically significant improvements in PASI response rates compared with placebo by week 4 of therapy. Response rates continued to increase through 24 weeks of therapy, after which they appeared to plateau. Approximately 60% of patients achieved a PASI-75 response at week 24, with a similar proportion at week 48.

Clinical data support the safety and tolerability of 12–24 weeks of etanercept treatment in patients with psoriasis [18, 25]. Published results from psoriasis clinical trials showed that etanercept was generally well tolerated. Adverse events observed during the initial 12-week placebo-controlled period were typically mild to moderate in intensity and occurred with similar frequency in etanercept and placebo groups [18, 25]. No apparent change in the adverse events profile occurred during the second 12-week period of the trials [18, 25].

Safety outcomes of the long-term Phase III psoriasis clinical trial, particularly for patients who received 50 mg twice weekly, have not yet been presented in detail; however, a preliminary analysis integrating data from the Phase II and 2 Phase III etanercept clinical trials, in which many patients received the 25-mg dosage, suggested that no new pattern of adverse events emerged during up to 60 weeks of treatment (A.B. Gottlieb et al., unpublished data presented at the 62nd annual meeting of the American Academy of Dermatology, 2004). Psoriasis clinical trials revealed no evidence of increased risk of opportunistic infections, tuberculosis, or skin cancers during up to 60 weeks of etanercept treatment (A.B. Gottlieb et al., unpublished data, 2004). Whereas

long-term data from clinical trials in many patients receiving etanercept for other indications, such as RA, support the general safety and tolerability of the 25-mg dosage, there have been infrequent cases of tuberculosis and rare cases of demyelinating disorders, pancytopenia, and congestive heart failure (Enbrel® [etanercept] package insert, European Medicines Agency; Enbrel [etanercept] summary of product characteristics; http://www.emea.eu.int/humandocs/Humans/EPAR/enbrel/enbrel.htm; 2005).

Infliximab

Infliximab, a chimeric monoclonal antibody that binds membrane-bound and soluble TNF-α, is still under evaluation for use in patients with psoriasis. Infliximab has demonstrated efficacy in psoriasis patients in randomized, placebo-controlled Phase II trials [2, 8]. However, the results of an international multicenter, randomized, placebo-controlled Phase III trial of adult patients with plaque psoriasis (baseline PASI ≥12 and ≥10% of BSA affected) were recently reported [26]. Patients were randomized to receive IV infliximab 5 mg/kg ($n=301$) or placebo ($n=77$) at initiation, week 2, and week 6 and then every 8 weeks through week 46. The placebo group crossed over to infliximab treatment at week 24, but the study remained blinded throughout. Patients who discontinued the study early because of lack or loss of response or for whom insufficient data were available were treated as not achieving the end points for analysis of the ITT population. At week 10 of treatment, the PASI-75 response rate was 80.4% in the infliximab-treated group and 2.6% in the placebo-treated group. This level of response was sustained through week 24. At week 50, the proportion of the ITT population that achieved PASI-75 dropped to 60.5%. However, excluding patients who missed 2 infusions, the PASI-75 rate at week 50 was 70.5% ($n=234$). The reason for the moderate reduction in efficacy is unclear but may be due to the development of inhibitory antibodies in a percentage of the patients.

The safety profile for infliximab during this extended treatment Phase III trial appears to be comparable to those observed during earlier studies [26]. The incidence of adverse events and serious adverse events in infliximab-treated patients was slightly elevated relative to those in placebo recipients through the first 24 weeks of treatment, and one patient who received infliximab died of sepsis. Elevations in aminotransferases were also observed in some infliximab-treated patients. In this trial, infection rates were comparable between the treated and placebo groups; however, like etanercept, postmarketing data from infliximab-treated patients with RA, Crohn's disease, or other indications for which infliximab is approved suggest a potential increased risk for events such as opportunistic infections (e.g., tuberculosis), lymphoma, or congestive heart failure.

Adalimumab

Adalimumab is a fully human monoclonal antibody that binds TNF-α. It is currently being evaluated in Phase III clinical studies for the treatment of moderate to severe plaque psoriasis. Results from a Phase II randomized, double-blind, placebo-controlled, multicenter clinical trial have been presented (K.B. Gordon et al., unpublished data presented at the 62nd annual meeting of the American Academy of Dermatology, 2004). Patients were randomized to receive placebo ($n=52$) or adalimumab, either an 80-mg first dose followed by 40 mg every other week (EOW; $n=46$) or an 80-mg first and second dose followed by 40 mg every week ($n=50$), for 12 weeks. In the preliminary analysis and presentation, 53 and 80% of the patients receiving adalimumab EOW or every week, respectively, achieved a PASI-75 response, compared to 4% of the patients receiving placebo ($n=52$). However, a careful reanalysis of the data suggested that the true efficacy was several percentage points lower (R.G.B. Langley et al., unpublished data, presented at the Fall European Academy of Dermatology and Venereology, 2005). Adverse events were similar to placebo, with headache, injection site pain, nausea, elevated triglycerides, cough, sinus congestion, and fatigue most common. Injection site pain was more frequent in the adalimumab 40-mg/week group (12%) than in the placebo group (6%).

Comparison of biological agents

In the absence of head-to-head clinical trials, it is difficult to accurately compare the efficacy of the biological agents for psoriasis. However, most of these agents use comparable evaluations of treatment efficacy. Alefacept is an exception, as it is used as remittive therapy; and alefacept data are presented using the as-treated population and "response at any time" analyses. Thus, although alefacept appears to maintain efficacy in responsive patients over multiple courses of treatment (Fig. 2), its efficacy cannot be readily compared with efalizumab, etanercept, or infliximab.

Available long-term data indicate that the improvements in psoriasis achieved during the first 12–24 weeks of efalizumab therapy are maintained or improved through at least 60 weeks of continuous treatment. Data support the efficacy of efalizumab through at least 36 months of continuous treatment. The efficacy of etanercept 25 mg administered twice weekly appears to be maintained through at least 48 weeks of continuous treatment. Preliminary findings of the PASI-75 response rate in patients who "step down" from etanercept 50 mg twice weekly to 25 mg twice weekly suggest that the initial improvements achieved with etanercept 50 mg twice weekly beyond what is observed with the 25-mg twice-weekly dose are slightly reduced following the recommended dose reduction. For infliximab, the very

high response rates obtained after 10 weeks of treatment appear to be sustained through 24 weeks but show a moderate reduction through 48 weeks with infusions every 8 weeks. Week 48 PASI-75 response rates were 62% for efalizumab-treated patients and approximately 45% for etanercept-treated patients (as-treated populations; Fig. 2). The PASI-75 response rate at week 50 for infliximab was 70.5% in patients who did not miss more than 1 infusion (Fig. 2).

The longer-term studies summarized here are not directly comparable because of differences in patient eligibility for initial enrollment and maintenance treatment and inclusion in efficacy analyses. For example, in contrast to the long-term etanercept study, patients in the efalizumab open-label trial were eligible for maintenance treatment only if they achieved a reasonable response during the first 12 weeks. This included the majority (94%) of patients completing the initial treatment phase. For the ITT efficacy analyses (presented through 12 weeks), all efalizumab-treated patients were included, even those who did not achieve PASI-50 or sPGA of mild, minimal, or clear at week 12 and were required to discontinue the trial. Direct data comparisons are further compromised by the available analyses. Efalizumab data are presented as both an ITT analysis for 48 weeks and an as-treated analysis for weeks 12 through 48. Etanercept data are presented as an ITT analysis through 48 weeks. Results from the long-term infliximab study are presented as an ITT analysis for the first 10 weeks of the trial and thereafter are presented as a "modified ITT" population, where missing data were considered as nonresponsive for patients who withdrew from the study for preselected reasons.

Given that psoriasis is a chronic, incurable disease, the availability of long-term data is critical for dermatologists evaluating treatment options. There is a paucity of long-term data derived from robust clinical trials for conventional nonbiological psoriasis therapies; therefore, dermatologists generally rely on treatment guidelines and personal experience in making their decisions about long-term administration of therapies such as methotrexate and cyclosporine. Because biologicals represent a new approach to the management of psoriasis, long-term data are of particular importance for this therapeutic class. Alefacept appears to be an effective therapy for inducing extended remissions in a small population of patients, and it has a demonstrated safety profile for remittive use. The long-term data available to date for efalizumab indicate that initial improvements in psoriasis are sustained for up to 36 months. Data from multiple clinical trials indicate that 22–41% of patients achieved PASI-75 in the first 3 months of therapy [6, 7, 16, 17, 24]; in an extended trial of patients receiving continuous therapy up to 36 months, 45.4% of the patients achieved PASI-75 at the 36-month end point (C.L. Leonardi et al., manuscript in preparation). Studies evaluating the long-term use of etanercept are under way, but only limited data are currently available, as suggested by the current 24-week limit for etanercept therapy in Europe. At 12 weeks, 49% of patients who received etanercept 50 mg twice weekly achieved PASI-75 [25]. Available data suggest that improvements are maintained for 48 weeks, as 60% of patients receiving 50 mg twice weekly achieved PASI-75 (S.K. Tyring et al., unpublished data presented at the summer meeting of the American Academy of Dermatology, 2005). Importantly, the safety data reported to date indicate that the safety profiles of efalizumab and etanercept allow for continuous long-term administration. Infliximab has demonstrated excellent response rates with induction and extended therapy, with an every-8-week infusion schedule optimal.

The approval of biological therapies, that is, the T-cell-modulators alefacept and efalizumab and the TNFα antagonist etanercept, along with the development of other TNFα antagonists such as infliximab and adalimumab, represent significant advances for the management of psoriasis. Their efficacy and safety profiles suggest that they may be suitable for remittive or continuous long-term administration, providing dermatologists with new options for the long-term management of their patients with psoriasis. The safety and efficacy data from ongoing clinical trials and the experience of dermatologists are important to define further how these agents can best be integrated into the psoriasis armamentarium.

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References

1. Carey W, Glazer S, Gottlieb AB et al (2006) Relapse, rebound, and psoriasis adverse events: an advisory group report. J Am Acad Dermatol 54(4 suppl 1):S171–S181
2. Chaudhari U, Romano P, Mulcahy LD, Dooley LT, Baker DG, Gottlieb AB (2001) Efficacy and safety of infliximab monotherapy for plaque-type psoriasis: a randomised trial. Lancet 357(9271):1842–1847
3. Ellis CN, Krueger GG (2001) Treatment of chronic plaque psoriasis by selective targeting of memory effector T lymphocytes. N Engl J Med 345:248–255
4. Gollnick HP (1996) Oral retinoids—efficacy and toxicity in psoriasis. Br J Dermatol 125 (suppl 49):6–17
5. Gollnick HP, Dummler U (1997) Retinoids. Clin Dermatol 15:799–810
6. Gordon KB, Papp KA, Hamilton TK et al (2003) Efalizumab for patients with moderate to severe plaque psoriasis: a randomized controlled trial. JAMA 290:3073–3080
7. Gottlieb AB, Gordon KB, Lebwohl MG et al (2004) Extended efalizumab therapy sustains efficacy without increasing toxicity in patients with moderate to severe chronic plaque psoriasis. J Drugs Dermatol 3:614–624
8. Gottlieb AB, Evans R, Li S et al (2004) Infliximab induction therapy for patients with severe plaque-type psoriasis: a randomized, double-blind, placebo-controlled trial. J Am Acad Dermatol 51:534–542
9. Gottlieb AB, Hamilton T, Caro I et al (2006) Long-term continuous efalizumab therapy in patients with moderate to severe chronic plaque psoriasis: updated results from an ongoing trial. J Am Acad Dermatol 54(4 suppl 1):S154–S163

10. Griffiths CE, Clark CM, Chalmers RJ, Li Wan PA, Williams HC (2000) A systematic review of treatments for severe psoriasis. Health Technol Assess 4:1–125
11. Griffiths CE, Dubertret L, Ellis CN et al (2004) Ciclosporin in psoriasis clinical practice: an international consensus statement. Br J Dermatol 150(suppl 67):11–23
12. Honigsmann H (2001) Phototherapy for psoriasis. Clin Exp Dermatol 26:343–350
13. Jullien D, Prinz JC, Langley RGB et al (2004) T-cell modulation for the treatment of chronic plaque psoriasis with efalizumab (Raptiva™): mechanisms of action. Dermatology 208:297–306
14. Krueger GG, Papp KA, Stough DB, Loven KH, Gulliver WP, Ellis CN (2002) A randomized, double-blind, placebo-controlled phase III study evaluating efficacy and tolerability of 2 courses of alefacept in patients with chronic plaque psoriasis. J Am Acad Dermatol 47:821–833
15. Lebwohl M, Christophers E, Langley R, Ortonne JP, Roberts J, Griffiths CE (2003) An international, randomized, double-blind, placebo-controlled phase 3 trial of intramuscular alefacept in patients with chronic plaque psoriasis. Arch Dermatol 139:719–727
16. Lebwohl M, Tyring SK, Hamilton TK et al (2003) A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. N Engl J Med 349:2004–2013
17. Leonardi CL, Papp KA, Gordon KB et al (2005) Extended efalizumab therapy improves chronic plaque psoriasis: results from a randomized phase III trial. J Am Acad Dermatol 52(3 Pt 1):425–433
18. Leonardi CL, Powers JL, Matheson RT et al (2003) Etanercept as monotherapy in patients with psoriasis. N Engl J Med 349:2014–2022
19. Menter A, Gordon K, Carey W et al (2005) Efficacy and safety observed during 24 weeks of efalizumab therapy in patients with moderate to severe plaque psoriasis. Arch Dermatol 141:31–38
20. Menter A, Kosinski M, Bresnahan BW, Papp KA, Ware JE Jr (2004) Impact of efalizumab on psoriasis-specific patient-reported outcomes. Results from three randomized, placebo-controlled clinical trials of moderate to severe plaque psoriasis. J Drugs Dermatol 3:27–38
21. Naldi L, Griffiths CE (2005) Traditional therapies in the management of moderate to severe chronic plaque psoriasis: an assessment of the benefits and risks. Br J Dermatol 152:597–615
22. Nickoloff BJ, Nestle FO (2004) Recent insights into the immunopathogenesis of psoriasis provide new therapeutic opportunities. J Clin Invest 113:1664–1675
23. Nijsten TE, Stern RS (2003) The increased risk of skin cancer is persistent after discontinuation of psoralen + ultraviolet A: a cohort study. J Invest Dermatol 121:252–258
24. Papp KA, Bressinck R, Fretzin S et al (2005) Safety of efalizumab in adults with chronic moderate to severe plaque psoriasis: a phase IIIb, randomized, controlled trial. Int J Dermatol [Epub ahead of print] Available at: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-4632.2006.02777.x
25. Papp KA, Tyring S, Lahfa M et al (2005) A global phase III randomized controlled trial of etanercept in psoriasis: safety, efficacy, and effect of dose reduction. Br J Dermatol 152:1304–1312
26. Reich K, Nestle FO, Papp K et al.; EXPRESS study investigators (2005) Infliximab induction and maintenance therapy for moderate-to-severe psoriasis: a phase III, multicentre, double-blind trial. Lancet 366:1367–1374
27. Roenigk HH Jr, Auerbach R, Maibach H, Weinstein G, Lebwohl M (1998) Methotrexate in psoriasis: consensus conference. J Am Acad Dermatol 38:478–485
28. Sterry W, Dubertret L, Papp K, Chimenti S, Larsen CG (2004) Efalizumab for patients with moderate to severe chronic plaque psoriasis: results of the international, randomized, controlled phase III clinical experience Raptiva (CLEAR) trial. J Invest Dermatol 123(2):A64
29. Yamauchi PS, Rizk D, Kormeili T, Patnaik R, Lowe NJ (2003) Current systemic therapies for psoriasis: where are we now? J Am Acad Dermatol 49(Suppl 2):S66–S77

ECM remodeling (Cox and Helvering, 2006) and wound healing (Ashcroft *et al.*, 2003; Mills *et al.*, 2005). It has been postulated that striae may result from an exaggerated wound healing process (Pinkus *et al.*, 1966), and, clinically — in pregnancy at least — the severity of striae appears to be related to a younger maternal age, although the age of onset of striae was not recorded by Salter *et al.* (2006). Of further interest is a significantly higher occurrence of varicosities in those individuals also presenting with striae (Salter *et al.*, 2006). It is plausible that these phenomena describe a generalized deterioration of ECM structures that could result from a number of biological events.

The study by Salter *et al.* (2006) presents an appealing relationship between the occurrence of skin striae and pelvic relaxation. It will be interesting to see whether prospective studies in this area can confirm whether clinical dermatological examination can identify, and therefore target therapy to, individuals at risk of prolapse in later life.

CONFLICT OF INTEREST

The author states no conflict of interest.

REFERENCES

Ashcroft GS, Mills SJ, Lei K, Gibbons L, Jeong MJ, Taniguchi M *et al.* (2003) Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor. *J Clin Invest* 111:1309–18

Bergfeld WF (1999) A lifetime of healthy skin: implications for women. *Int J Fertil Womens Med* 44:83–95

Cox DA, Helvering LM (2006) Extracellular matrix integrity: a possible mechanism for differential clinical effects among selective estrogen receptor modulators and estrogens? *Mol Cell Endocrinol* 247:53–9

Lee KS, Rho YJ, Jang SI, Suh MH, Song JY (1994) Decreased expression of collagen and fibronectin genes in striae distensae tissue. *Clin Exp Dermatol* 19:285–8

Mills SJ, Ashworth JJ, Gilliver SC, Hardman MJ, Ashcroft GS (2005) The sex steroid precursor DHEA accelerates cutaneous wound healing via the estrogen receptors. *J Invest Dermatol* 125:1053–62

Olsen AL, Smith VJ, Bergstrom JO, Colling JC, Clark AL (1997) Epidemiology of surgically managed pelvic organ prolapse and urinary incontinence. *Obstet Gynecol* 89:501–6

Phillips CH, Anthony F, Benyon C, Monga AK (2006) Collagen metabolism in the uterosacral ligaments and vaginal skin of women with uterine prolapse. *BJOG* 113:39–46

Pinkus H, Keech MK, Mehregan AH (1966) Histopathology of striae distensae, with special reference to striae and wound healing in the Marfan syndrome. *J Invest Dermatol* 46:283–92

Salter SA, Batra RS, Rohrer TE, Kohli N, Kimball AB (2006) Striae and pelvic relaxation: two disorders of connective tissue with a strong association. *J Invest Dermatol* 126:1745–8

Sheu HM, Yu HS, Chang CH (1991) Mast cell degranulation and elastolysis in the early stage of striae distensae. *J Cutan Pathol* 18:410–6

Uebersax JS, Wyman JF, Shumaker SA, McClish DK, Fantl JA (1995) Short forms to assess life quality and symptom distress for urinary incontinence in women: the Incontinence Impact Questionnaire and the Urogenital Distress Inventory. *Neurourol Urodyn* 14:131–9

Ulmsten U, Ekman G, Giertz G, Malmstrom A (1987) Different biochemical composition of connective tissue in continent and stress incontinent women. *Acta Obstet Gynecol Scand* 66:455–7

Viennet C, Bride J, Armbruster V, Aubin F, Gabiot AC, Gharbi T *et al.* (2005) Contractile forces generated by striae distensae fibroblasts embedded in collagen lattices. *Arch Dermatol Res* 297:10–7

Watson REB, Parry EJ, Humphries JD, Jones CJP, Polson DW, Kielty CM *et al.* (1998) Fibrillin microfibrils are reduced in skin exhibiting striae distensae. *Br J Dermatol* 138:931–7

See related article on pg 1784

Signaling Downstream of p38 in Psoriasis

J. Simon C. Arthur[1] and Joanne Darragh[1]

Psoriasis is an inflammatory skin disease characterized by infiltration of the skin by T cells and increased production of pro-inflammatory cytokines. Two recent reports show that the p38-activated kinases mitogen-activated protein kinase-activated protein kinase 2 and mitogen- and stress-activated protein kinase are activated in psoriatic skin and may contribute to the production of pro-inflammatory cytokines.

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Psoriasis is a common skin disorder, and estimates of its prevalence vary from 0.5% to 4.6% of the world population. A significant proportion of patients may also exhibit psoriatic arthritis, although the onset of arthritis can precede the development of psoriasis. Psoriasis is an inflammatory disorder that is characterized by epidermal hyperplasia, increases in keratin expression, and recruitment of T cells as well as changes in the endothelial vascular system (Bos *et al.*, 2005; Bowcock and Krueger, 2005; Krueger and Ellis, 2005). On the basis of the analysis of the cytokines produced, psoriasis is often considered to be a T-helper 1 condition, as levels of IFN-γ, tumor necrosis factor (TNF), IL-1, IL-2, IL-6, and IL-8 are increased in psoriatic lesions. The recruitment of T cells is likely to be critical to the development of the disease; however, the immunology of psoriasis is complex, and several other cell types, including dendritic cells, phagocytes, and keratinocytes, are also likely to play a role (Bos *et al.*, 2005; Bowcock and Krueger, 2005; Krueger and Ellis, 2005).

Recent advances in treatment have confirmed a role for T cells in psoriasis, and agents that target T cells, such as alefacept and efalizumab, have been shown to reduce the symptoms of psoriasis. Interestingly, anti-TNF therapies have also proved effective at treating psoriasis, demonstrating a critical role for this cytokine in the condition (Bos *et al.*, 2005; Bowcock and Krueger, 2005;

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Krueger and Ellis, 2005). The source of TNF in psoriasis is, however, unclear, as several cell types, including both T cells and keratinocytes, have the potential to produce TNF in the skin. The signaling pathways that mediate both the production and the pathological effects in psoriatic skin are also not clearly defined.

Mitogen-activated protein kinases (MAPKs) form a series of conserved signaling networks in mammalian cells (Roux and Blenis, 2004). Four main groups of MAPKs have been identified: the classical MAPKs extracellular signal-regulated kinase 1 (ERK1) and ERK2; c-Jun N-terminal kinases (JNKs); p38 MAPKs; and atypical MAPKs such as ERK5 and ERK3. Of these, both the ERK1/2 and p38 MAPK cascades have been implicated in the production of TNF, and, in addition, these cascades are also activated in many cell types in response to TNF signaling. p38 MAPKs have also attracted particular interest in the immune system, as p38 was originally identified as the cellular target of a group of drugs that inhibited pro-inflammatory cytokine production in response to lipopolysaccharide. This has led several companies to develop and patent p38 inhibitors as potential treatments for several inflammatory diseases, including arthritis and psoriasis.

Analysis of psoriatic skin has shown that the levels of both ERK1/2 and p38 activity are increased, which is consistent with a potential role for these kinases in the development of psoriasis (Johansen *et al.*, 2005). Both the ERK1/2 and p38 MAPK cascades are able to activate additional downstream kinases: ERK1/2 activates ribosomal S6 kinase, p38α activates MAPKAP-K2, and both ERK1/2 and p38α are able to activate mitogen- and stress-activated protein kinase 1 (MSK1) and MSK2 (Roux and Blenis, 2004). Two recent reports have now also shown that at least two of these downstream kinases, MAPKAP-K2 and MSK1, are activated in psoriatic skin.

Studies on MAPKAP-K2 knockout mice have identified a role for this kinase in the production of some pro-inflammatory cytokines in response to lipopolysaccharide, notably TNF, IL-6, IL-1β, and IFN-γ (Kotlyarov *et al.*, 1999). Levels of these cytokines in MAPKAP-K2 knockout mice were significantly decreased as compared with those in wild-type controls in response to lipopolysaccharide. MAPKAP-K2 is thought to control cytokine production at a post-transcriptional level, both via the regulation of RNA stability, as happens for IL-6, and by control of protein translation, as occurs for TNF (Kotlyarov *et al.*, 1999; Neininger *et al.*, 2002). In a recent paper in the *Journal of Immunology*, Johansen *et al.* (2006) have shown that TNF protein levels, but not those of the TNF mRNA, are increased in psoriatic skin. The levels of MAPKAP-K2 activity, as judged by phospho-blotting, were also increased in psoriatic skin, suggesting a possible mechanism for the elevated TNF levels. Consistent with this, it was found that small interfering RNA (siRNA)-mediated knockdown of MAPKAP-K2 reduced the production of TNF in primary keratinocytes stimulated with IL-1β (Johansen *et al.*, 2006). It would therefore be of interest to see whether knockout of MAPKAP-K2 results in a protective effect in mouse models of skin inflammation.

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MSK1, and the related kinase MSK2, are nuclear kinases activated downstream of both ERK1/2 and p38α (Darragh *et al.*, 2005). Double knockouts of both MSK1 and MSK2 are viable (Wiggin *et al.*, 2002) and do not exhibit an overt phenotype. Cells from these mice have, however, established a role for these kinases in the mitogen- and stress-induced phosphorylation of the transcription factors cAMP-response element-binding protein (CREB) and activation transcription factor 1 and the chromatin proteins histone H3 and HMG-14 (Soloaga *et al.*, 2003; Wiggin *et al.*, 2002). Downstream of this, it has been shown that MSKs contribute to the induction of some immediate early genes, including c-fos, nur77, and nor1 (Darragh *et al.*, 2005). The role of MSK has not, however, been specifically addressed in the immune system of these mice. In a paper in this issue of the *Journal of Investigative Dermatology*, Funding *et al.* (2006) demonstrate that MSK1 activity, as judged by phospho-blotting, is increased in psoriatic compared with normal skin. Immunostaining experiments suggested that this activation of MSK occurred mainly in keratinocytes in the psoriatic skin.

Using primary keratinocytes, Funding *et al.* (2006) further show that IL-1β activates p38α, which in turn activates MSK1, leading to CREB phosphorylation. Consistent with the work in knockout mice, siRNA-mediated knock-down of MSK1 resulted in decreased CREB phosphorylation. Given the activation of MSK1 in psoriatic skin, it would be of interest for future work to determine whether CREB phosphorylation is also increased in psoriatic skin. The siRNA knock-down of MSK1 also resulted in modest decreases in IL-6 and IL-8 production. Interestingly, a role for MSK1 in IL-6 transcription in fibroblasts has been suggested previously. Specifically, MSK was suggested to act either upstream of NF-κB or by inducing chromosomal remodeling at the IL-6 locus (Vermeulen *et al.*, 2003). Although the reduction in cytokine production in the study by Funding *et al.* (2006) was modest, it is worth noting that only MSK1 was targeted by siRNA. Evidence from knockout mice has suggested that in most cell types MSK1 and MSK2 are able to functionally compensate for each other, and therefore knockout of both isoforms would be needed to show the maximal effect on cytokine production (Wiggin *et al.*, 2002). Future work to address the role of MSKs in cytokine production, possibly using gene-targeted mice, would therefore be very interesting.

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES

Bos JD, de Rie MA, Teunissen MB, Piskin G (2005) Psoriasis: dysregulation of innate immunity. *Br J Dermatol* 152:1098–1107

Bowcock AM, Krueger JG (2005) Getting under the skin: the immunogenetics of psoriasis. *Nat Rev Immunol* 5:699–711

Darragh J, Soloaga A, Beardmore VA, Wingate A, Wiggin GR, Peggie M *et al.* (2005) MSKs are required for the transcription of the nuclear orphan receptors Nur77, nurr1 and nor1 downstream of MAP kinase signalling. *Biochem J* 390:749–59

Funding AT, Johansen C, Kragballe K, Otkjær K, Jensen UB, Madsen MW *et al.* (2006) Mitogen- and stress-activated protein kinase 1 is activated

in lesional psoriatic epidermis and regulates the expression of pro-inflammatory cytokines. *J Invest Dermatol* 126:1784–91

Johansen C, Funding AT, Otkjaer K, Kragballe K, Jensen UB, Madsen M *et al.* (2006) Protein expression of TNF-α in psoriatic skin is regulated at a posttranscriptional level by MAPK-activated protein kinase 2. *J Immunol* 176:1431–8

Johansen C, Kragballe K, Westergaard M, Henningsen J, Kristiansen K, Iversen L (2005) The mitogen-activated protein kinases p38 and ERK1/2 are increased in lesional psoriatic skin. *Br J Dermatol* 152:37–42

Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD *et al.* (1999) MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. *Nat Cell Biol* 1:94–7

Krueger G, Ellis CN (2005) Psoriasis: recent advances in understanding its pathogenesis and treatment. *J Am Acad Dermatol* 53(Suppl 1): S94–S100

Neininger A, Kontoyiannis D, Kotlyarov A, Winzen R, Eckert R, Volk HD *et al.* (2002) MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. *J Biol Chem* 277:3065–8

Roux PP, Blenis J (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 68:320–44

Soloaga A, Thomson S, Wiggin GR, Rampersaud N, Dyson MH, Hazzalin CA *et al.* (2003) MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *EMBO J* 22:2788–97

Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G (2003) Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO J* 22:1313–24

Wiggin GR, Soloaga A, Foster JM, Murray-Tait V, Cohen P, Arthur JS (2002) MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. *Mol Cell Biol* 22:2871–81

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Plenty New Under the Sun

Jonathan Rees[1]

Variation at the melanocortin 1 receptor (*MC1R*) is very common in most non-African world populations. A range of variants predispose to skin cancer, including melanoma. What remains unclear are the mechanisms linking gene variation with sun sensitivity or tumor risk. In particular, it remains unclear whether pigmentary effects of the *MC1R* can account for all of the increase in cancer risk.

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The modern study of the genetics of normal human pigmentary variation is just over ten years old (Rees, 2003). The field may not yet be mature, but there is a clear feeling that the first few chapters of this particular story can now be written: in broad outline we now know which genes account for most variation in human skin and hair color. The paper by Stratigos and colleagues (2006, this issue), in which melanocortin 1 receptor (*MC1R*) variants have been studied in relation to pigmentary phenotype and melanoma, is a welcome addition to the literature — welcome, because most previous studies of pigmentary genetics have concentrated on northern rather than southern European populations, and because the paper highlights some of the uncertainties in our understanding of the mechanisms by which *MC1R* variation affects phenotype.

The first modern study of the genetics of red hair was carried out almost a century ago by the Davenports at what was then the Carnegie Institute and is now famous worldwide as Cold Spring Harbor Laboratory (reviewed by Rees, 2003). The Davenports studied kindreds with red hair and suggested that the red-hair trait approximated to an autosomal recessive. They were right, although not all subsequent work came to the same conclusion. The next seminal advance, following a few unsuccessful attempts at mapping the red-hair trait in humans, came from the marriage of mouse and molecular genetics. The genes underlying a range of murine coat-color mutations were cloned, and within years the genetic bases for some rare mendelian pigmentary syndromes in humans were identified. One gene, the melanocortin 1 receptor, the subject of the study by Stratigos *et al.* (2006), was cloned in mice by Roger Cone's group in 1993 (Robbins *et al.*, 1993). Work in mice showed that signaling through this pathway led to an increase in the ratio of eumelanin to pheomelanin in hair (Robbins *et al.*, 1993). Conversely, the absence of, or a reduction in, signaling, whether due to mutation at the *mc1r* or absence of the ligand α-melanocyte-stimulating hormone, resulted in a relative overproduction of pheomelanin, leading to a yellow coat.

Shortly after Cone and colleagues' work in mice, it was reported that most red-haired persons harbored homozygous diminished-function alleles at the *MC1R* (Valverde *et al.*, 1995). The human *MC1R* codes for a 317-amino acid G-coupled receptor, and many non-African human populations show a striking degree of variation at this locus (Wong and Rees, 2005). Over 75 different *MC1R* alleles in humans have been identified (Wong and Rees, 2005), and Stratigos *et al.* (2006) show that even in a southern European population 38% of the control population shows *MC1R* variants. A striking feature of the *MC1R* is that a large number of the alleles appear to be quantitatively different in terms of function; that is, rather than all the alleles associated with red hair being complete loss-of-function alleles, they show varying degrees of signaling activity (Ringholm *et al.*, 2004). Given that there is evidence for a clear additive (or dosage) effect among 0, 1, and 2 variant alleles, a range of physiological activity can arise from a single locus. *MC1R* heterozygotes are intermediate for hair eumelanin and pheomelanin ratios, and those with different shades of red hair appear to differ at the *MC1R* (Naysmith *et al.*, 2004). Thus, although red hair approximates to an autosomal recessive trait, the more closely one studies the phenotype, the more subtle the relation between particular alleles and

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TNF Inhibition Rapidly Down-Regulates Multiple Proinflammatory Pathways in Psoriasis Plaques[1]

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The mechanisms of action of marketed TNF-blocking drugs in lesional tissues are still incompletely understood. Because psoriasis plaques are accessible to repeat biopsy, the effect of TNF/lymphotoxin blockade with etanercept (soluble TNFR) was studied in ten psoriasis patients treated for 6 months. Histological response, inflammatory gene expression, and cellular infiltration in psoriasis plaques were evaluated. There was a rapid and complete reduction of IL-1 and IL-8 (immediate/early genes), followed by progressive reductions in many other inflammation-related genes, and finally somewhat slower reductions in infiltrating myeloid cells ($CD11c^+$ cells) and T lymphocytes. The observed decreases in IL-8, IFN-γ-inducible protein-10 (CXCL10), and MIP-3α (CCL20) mRNA expression may account for decreased infiltration of neutrophils, T cells, and dendritic cells (DCs), respectively. DCs may be less activated with therapy, as suggested by decreased IL-23 mRNA and inducible NO synthase mRNA and protein. Decreases in T cell-inflammatory gene expression (IFN-γ, STAT-1, granzyme B) and T cell numbers may be due to a reduction in DC-mediated T cell activation. Thus, etanercept-induced TNF/lymphotoxin blockade may break the potentially self-sustaining cycle of DC activation and maturation, subsequent T cell activation, and cytokine, growth factor, and chemokine production by multiple cell types including lymphocytes, neutrophils, DCs, and keratinocytes. This results in reversal of the epidermal hyperplasia and cutaneous inflammation characteristic of psoriatic plaques. *The Journal of Immunology,* **2005, 175: 2721–2729.**

The TNF inhibitors are approved for use across a range of inflammatory disorders including rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, and psoriasis. Despite the fact that >500,000 patients have been treated with TNF-blocking drugs worldwide, the anti-inflammatory mechanisms of action of these agents in lesional tissue are incompletely understood. Most of the data in rheumatoid arthritis have been generated from studying circulating cells or proteins and inferring what may be happening in the synovium. The paucity of data is in part explained by the relative inaccessibility of the gastrointestinal tract and joints to repeated biopsy. The consequence of this lack of knowledge is that the reasons for the different efficacy and safety spectra of the TNF blockers (infliximab, adalimumab, and etanercept) are not well understood.

TNF has complicated effects on both cell differentiation and expression of inflammatory genes (1–4). Despite the fact that many data have been generated in various cultured cell types, these in vitro models are not necessarily good models of interacting cell types involved in a tissue-specific autoimmune process. For example, genes induced by TNF are specific to the cell type and state of differentiation of the cell studied. Hence, there may be effects of TNF that are undiscovered in complicated cellular mixes of inflammatory diseases or which cannot be studied well in model systems. The recent approval of etanercept for psoriasis offers an opportunity to study in situ mechanisms of action because the skin is a relatively convenient and accessible tissue to study.

Psoriasis is a life-disabling disorder characterized by the presence of scaly, red, raised cutaneous lesions, which are widespread in ~25% of afflicted patients. Psoriatic plaques are histologically recognized by typical patterns of abnormal epidermal hyperplasia and differentiation, similar to that seen at the edges of acute and nonhealing wounds (regenerative maturation) (5, 6). However, in the past 20 years, it has become clear that these epidermal changes are secondary to robust immune activation within psoriatic plaques characterized by increased numbers and activation of both T lymphocytes and dendritic cells (DCs)[3] (professional APCs) (7–15). Animal models demonstrate a role for T cell activation, local cutaneous T cells, and TNF (16–18). Clinically, agents that specifically block T cells or TNF clear psoriasis (19–24). TNF blockade induced by either etanercept, a form of soluble p75 TNF receptor that binds both TNF and lymphotoxin (LT), or infliximab, a chimeric, monoclonal anti-TNF Ab, clears psoriasis. This clinical remission is associated with substantially decreased numbers of intraepidermal T cells and by normalization of epidermal proliferation and differentiation, as measured by decreased epidermal thickness and normalized protein expression of keratin 16 (K16) (20). It is not known whether the clinical and histological remission induced by TNF/LT blockade is due to effects on T cells, DCs,

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[3] Abbreviations used in this paper: DC, dendritic cell; IP-10 (CXCL10), interferon-γ-inducible protein-10; iNOS, inducible nitric oxide synthase; K16, keratin 16; LT, lymphotoxin; MMP, matrix metalloproteinase; MIG, monokine induced by IFN-γ; PASI, Psoriasis Area and Severity Index; RS, response score; HARP, human acidic ribosomal protein.

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or both. Etanercept is an ideal agent with which to study the effect of TNF blockade on cellular immune regulation in plaques because its actions are thought to be due to neutralization of TNF and not to depletion of cells bearing cell surface TNF.

Materials and Methods

Patient studies

Adult patients with moderate to severe psoriasis were treated with etanercept monotherapy (25 mg s.c. twice weekly) for 24 wk under a protocol approved by the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School Institutional Review Board. At the time this study was performed, the 25-mg twice-weekly dose was the only Food and Drug Administration-approved dose of etanercept for adults with psoriasis. Systemic and phototherapies were excluded for 1 mo before dosing, and topical medications were excluded for 2 wk before dosing. Eucerin cream (Beiersdorf) was the standard moisturizer used throughout the study but was not applied before study evaluations. Clinical efficacy was assessed using the Psoriasis Area and Severity Index (PASI) (25). At baseline, biopsies were taken from uninvolved skin and an index psoriasis lesion. Repeat biopsies were taken from the index lesion after 1, 3, and 6 mo of etanercept treatment. Our tissue-based analysis sought to determine the extent to which etanercept improved or reversed disease-defining pathology at different time points, and also how disease improvement correlated with suppression of specific inflammatory leukocyte subsets or inflammatory gene products. Laboratory-based evaluators were blinded to the clinical results until all data were collected.

Immunohistochemistry

Tissue sections were stained with hematoxylin (Fisher) and eosin (Shandon) (H&E) and with mouse anti-human mAbs to elastase (Dako), K16 (Sigma-Aldrich), ICAM-1 (BD Pharmingen), CD3 (BD Biosciences), and CD11c (BD Pharmingen). A secondary biotin-labeled horse anti-mouse Ab (Vector Laboratories) was amplified with the avidin-biotin complex (Vector Laboratories). 3-Amino-9-ethylcarbazole (Sigma-Aldrich) was the chromogen used. Epidermal thickness measures and cell counts (per mm per ×10 field) were determined using computer-assisted image analysis (Image Pro-Plus (Media Cybernetics)). K16 and ICAM-1 protein expression were quantitated on a 0–4 scale, with 0 indicating a return to a normal staining pattern. Absence of suprabasal staining for K16 keratin is the normal pattern seen in normal or uninvolved skin and is assigned a score of 0.

Tissue mRNA gene expression

RNA was extracted from skin biopsies frozen in liquid nitrogen using the RNeasy Mini Kit (Qiagen). The RT-PCR was performed using EZ PCR core reagents and primers and probes (Applied Biosystems) as previously published (26). The primer sequences have been published for K16, inducible nitric oxide synthase (iNOS), IL-8, IFN-γ, STAT-1, IL-12/IL-23p40, IL-23p19, monokine induced by IFN-γ (MIG), and human acidic ribosomal protein (HARP) (26). Sequences of the other primers used for this study are: IL-1β forward, GCACGATGCACCTGTACGAT; IL-1β reverse, AGACATCACCAAGCTTTTTTGCT, IL-1β probe, 6FAM-CTGAACTGCACGCTCCGGGACTC-TAMRA (GenBank accession number NM_000576); IL-6 forward, CCAGGAGCCCAGCTATGAAC; IL-6 reverse, CCCAGGGAGAAGGCAACTG, IL-6 probe, 6FAM-CC TTCTCCACAAGCGCCTTCGGT-TAMRA (GenBank accession number NM_000600); MIP-3α (CCL20) forward, GCTTTGATGTCAGTGCTGC TACTC; MIP 3α reverse, GTATCCAAGACAGCAGTCAAAGTTG; MIP-3α probe, 6FAM-TGCGGCGAATCAGAAGCAGCAA-TAMRA (GenBank accession number NM_004591); matrix metalloproteinase (MMP)-12 forward, AGCACTTCTTGGGTCTGAAAGTG; MMP-12 reverse; CGAGGTGCGTGCATCATCT; MMP-12 probe, 6FAM-CCGGG CAACTGGACACATCTACCC-TAMRA (GenBank accession number L23808); IFN-γ-inducible protein-10 (IP-10/CXCL10) forward, TCC ACGTGTTGAGATCATTGC; IP-10 reverse, AATTCTTGATGGCCT TCGATTC; IP-10 probe, 6FAM-ACAATGAAAAAGAAGGGTGAGAA GAGATGTCTGAA-TAMRA (GenBank accession number NM_001009191); CD83 forward, TCGACGCCCCCAATGA; CD83 reverse, CCCCGAGTTG CAGCTGG; CD83 probe, 6FAM-AGGCCCTATTCCCTGAAGATC CGAAACA-TAMRA (GenBank accession number NM_004233); granzyme B forward, GAGGCCCTCTTGTGTGTAACAAG; granzyme B reverse, CAGGCTCGTGGAGGCATG; granzyme B probe, 6FAM-CCAGGGCAT TGTCTCCTATGGACGAA-TAMRA (GenBank accession number NM_004131); IL-19 forward, CATGCAACTCTATTCCCAGCTACTT; IL-19 reverse, AGGTCAAAGCTGCAGTGAGCCATGATTG; IL-19 probe, 6FAM-GGGTGTCTCAATCTGGCACC-TAMRA (GenBank accession number, AF276915); TNF-α forward, CCCAGGCAGTCAATCATCTTC; TNF-α reverse, GCTTGAGGGTTTGCTACAACA; TNF-α probe 6FAM-CGAACCCCGAGTGACAAGCCTGT-TAMRA (GenBank accession number NM_000594).

Immunofluorescence

Frozen lesional tissue sections from psoriasis patients ($n = 4$) were fixed in acetone and treated with 10% normal horse serum. For CD11c and iNOS colocalization experiments, sections were initially incubated overnight with purified CD11c (BD Pharmingen) (1/100) and then secondary Ab Alexa fluor 488 goat anti-mouse IgG1 (Molecular Probes) for 30 min (1/250). iNOS (R&D Systems) conjugated with Alexa fluor 546 (Molecular Probes) was then incubated with these CD11c-stained slides for 2 h (1/500). Images were acquired using appropriate filters of a Zeiss Axioplan 2I microscope with Plan Apochromat 20 ×0.7NA lens and a Hamamatsu Photonics model C4742 "Orca" ER-cooled charge-coupled device camera, controlled by Universal Imaging MetaVue software.

Statistical analysis

Cell counts and gene expression changes were computed by Student's t test, and significance was accepted as $p < 0.05$. U statistics (U score) were developed to rank patient responses by combining percent change in epidermal thickness and the presence or absence of K16 immunostaining into a response score (RS), as previously published (26, 27). This mathematical model describes psoriasis disease activity across the range of complete disease resolution to highly active disease present and stratifies patient responses from best (lowest score) to worst (highest score). Changes in inflammatory cells or genes, either singly or in combinations, were ranked by a similar U score stratification method, and comparison of multiples of up to three genes at a time was designated a pathway score. We ordered changes in individual patients by U scores, because nonparametric statistical methods make the fewest assumptions about quantitative cause/effect relationships. Correlations between the RS and lesional cell counts, gene expression, or the pathway score were then determined, and the r score is indicated for the given comparisons.

Results

Clinical and histological responses

In this study of 10 patients with moderate to severe psoriasis vulgaris, etanercept decreased the PASI by a mean of 29% (range, 5–80% decrease) after 1 month treatment ($p < 0.02$), and 57% (range, 24–94% decrease) after 3 mo of treatment ($p < 0.01$). At 3 mo of treatment, 6 of 10 patients attained a PASI 50 response, whereas at 6 mo of treatment 6 of 10 patients attained a PASI 75 response. The time course and extent of improvement seen in patients in this trial are thus similar to outcomes seen in larger, blinded clinical trials (22, 24).

The effects of etanercept on disease histopathology, expression of K16 (immunohistochemistry and quantitative mRNA measures), and ICAM-1 expression in epidermal keratinocytes are illustrated in Fig. 1. In general, progressive reversal of epidermal acanthosis and psoriasiform rete elongation was seen during 6 mo of treatment, although near maximal improvement was noted in a few cases after 3 mo of treatment. After 6 mo of treatment, thinning of the epidermis and normalization of keratinocyte differentiation occurred in 9 of 10 subjects, and absence of K16 in suprabasal keratinocytes was noted in 8 of 10 cases. Likewise, expression of ICAM-1 by epidermal keratinocytes was eliminated in 8 of 10 cases after 6 mo of treatment. We consider that histological remission of psoriasis was attained in these 8 patients. The progression in disease improvement during 6 mo of treatment can be appreciated from the overall histopathology and from quantitative measures of epidermal thickness and K16 mRNA in biopsy specimens (Fig. 1).

Etanercept decreases myeloid DCs and T cells in plaques

CD11c is a marker for a group of DCs with very large increases in the epidermis and dermis of psoriasis lesions (26, 28, 29). Both

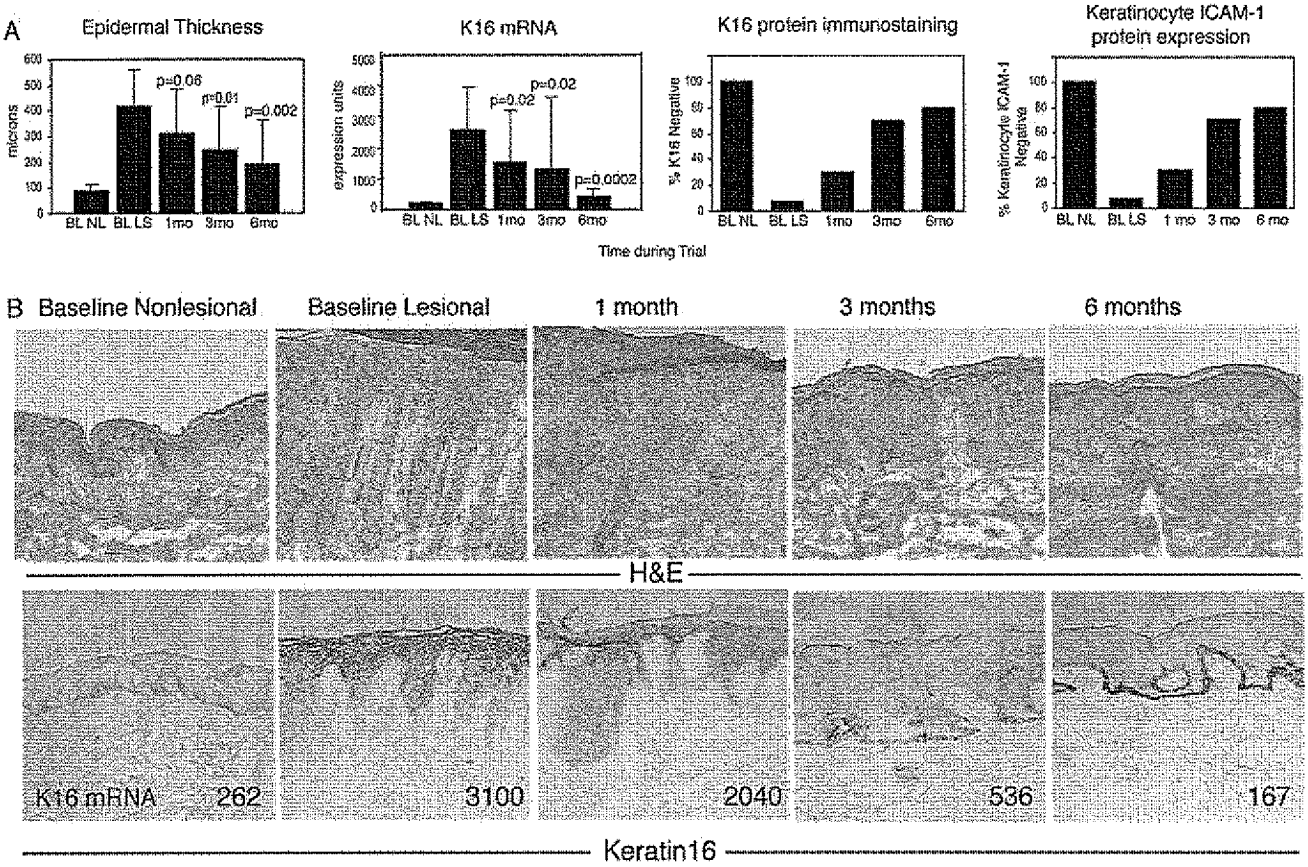

FIGURE 1. Response to etanercept treatment. *A*, Decreased epidermal thickness, K16 mRNA (normalized to HARP), K16 protein, keratinocyte ICAM-1 at baseline nonlesional (BL NL), baseline lesional (BL LS), 1, 3, and 6 mo of treatment. K16 and ICAM-1 protein staining quantitated on a 0–4 scale. *B*, Representative photomicrographs of H&E- and K16-stained frozen skin sections from a responding, etanercept-treated patient showing gradual reversal of psoriasis features (×10). K16 mRNA value (normalized to HARP) for this patient is indicated at the bottom of the photomicrograph, showing direct relationship to protein immunostaining. There is progressive reduction in K16 mRNA in association with a decrease in K16 protein in this individual patient. Comparison of results in treatment groups to lesional results, p value indicated.

$CD11c^+$ DCs and $CD3^+$ T cells were highly increased in pretreatment plaques compared with uninvolved skin (Fig. 2). Etanercept treatment resulted in progressive decreases in total $CD3^+$ and $CD11c^+$ cell populations (Fig. 2) as well as pathological epidermal thickness (Fig. 1). However, for both DCs and T cells, decreases in intraepidermal cell numbers were more marked than in dermal cell numbers. For example, the mean decrease in epidermal $CD11c^+$ cells was 95% at 6 mo vs 60% for dermal $CD11c^+$ cells. Additionally, the decrease in intraepidermal DCs was greater than that of intraepidermal T cells (95% vs 74%, respectively).

To describe overall improvement in psoriasis at the various analysis time points, we used a new multivariate tool that generates a RS by integrating epidermal thickness, K16 mRNA levels, and K16 protein in suprabasal keratinocytes (27). This mathematical model describes psoriasis disease activity across the range of complete disease resolution to highly active disease present, and stratifies patient responses from best (lowest score) to worst (highest score). The RS, which is a multivariate U score, can then be used to establish the extent to which a given degree of improvement is related to modulation of inflammatory cell types or inflammatory gene products in that biopsy. Overall, we found that reversal of epidermal hyperplasia (quantified as the RS) was more highly correlated with reductions in $CD11c^+$ cells than T cells, especially for infiltration of the epidermis by these cell subsets (Fig. 3). Taken together, these observations suggest that inflammatory leukocytes may act locally in the epidermis to produce the psoriatic phenotype and that DCs may play a larger role than previously suspected.

Effects on expression of inflammation-related genes

Table I lists numerous inflammation-related gene products that are increased in psoriasis lesions and that were assessed by real time RT-PCR during etanercept-induced disease improvement (30–35). These products include early genes induced by TNF in model systems (Il-1β, IL-8, MIP-3α, IL-6), type 1 pathway genes broadly related to IFN-regulated responses (IL-23, STAT-1, MIG, iNOS, IP-10, IL-8) (36, 37), and genes typically activated in myeloid cells (iNOS, IL-19, MMP-12, IL-23, CD83) vs lymphocytes (granzyme B, IFN-γ). In previous work, we have proposed this type 1 pathway of gene activation, which may control pathogenic inflammation in psoriasis lesions. According to this model, several upstream cytokines (including IL-23, IFN-γ, TNF) induce expression of end stage inflammatory genes such as iNOS or IL-8 (36). These gene groups are not mutually exclusive, e.g.: IL-8 is an early response gene but can also be a secondary product of the type 1 pathway; iNOS is produced by myeloid DCs but is also a product of the type 1 pathway.

Fig. 4 illustrates expression of each of these gene products (normalized to HARP) in uninvolved skin vs psoriatic skin lesions at baseline and after 1, 3, and 6 mo of etanercept treatment. Two basic patterns of gene regulation by etanercept are seen. IL-1 and

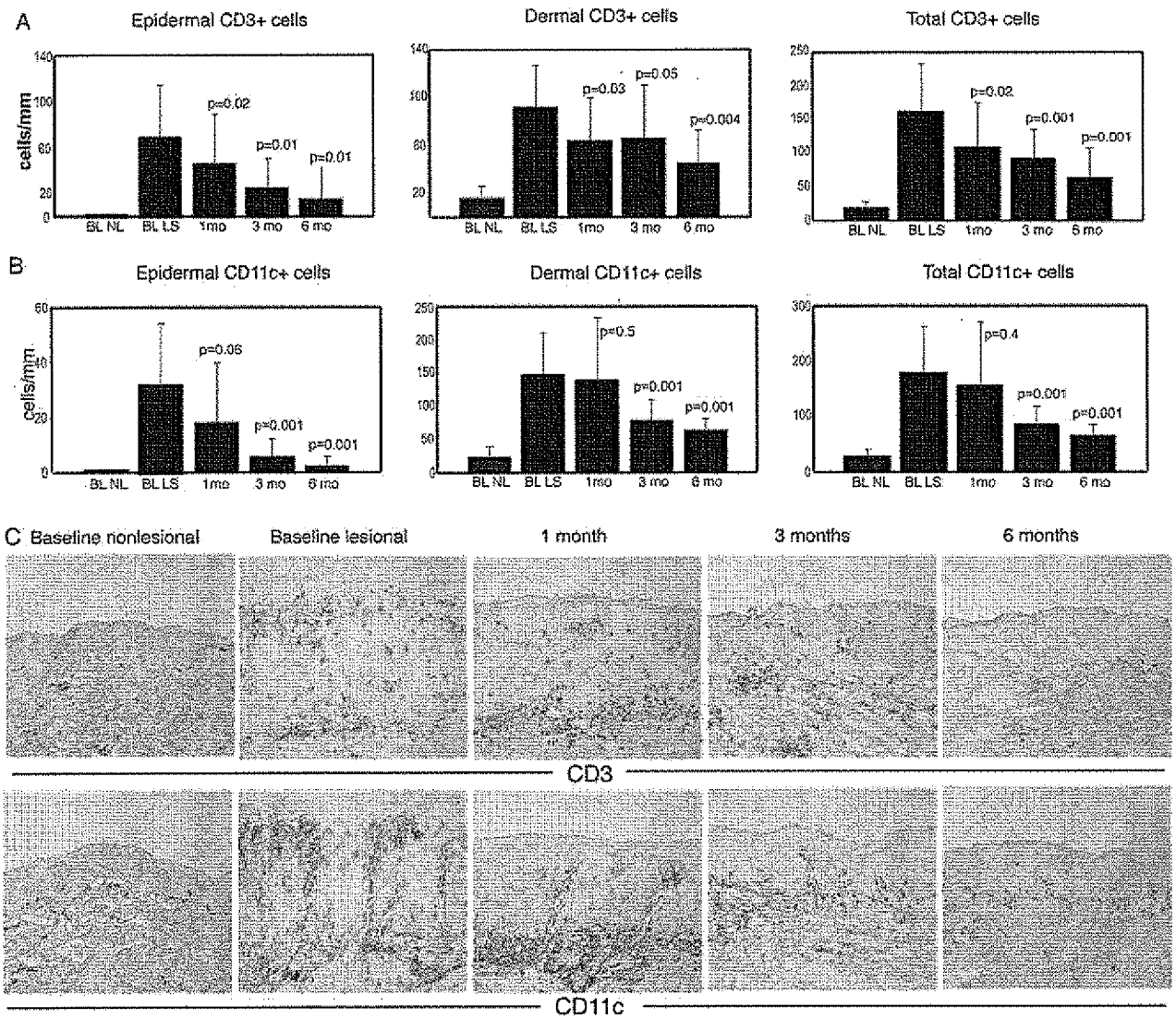

FIGURE 2. Decreased cellular infiltration in epidermis and dermis of psoriasis plaques in response to etanercept treatment. $CD3^+$ cells (*A*) and $CD11c^+$ cells (*B*) at baseline nonlesional (BL NL), baseline lesional (BL LS), and 1, 3, and 6 mo of treatment. *C*, Representative photomicrographs of $CD3^+$ and $CD11c^+$ immunostained frozen skin sections from a patient responding to etanercept, showing gradual reversal of $CD3^+$ and $CD11c^+$ cells infiltrating psoriasis lesions (×10). Comparison of results in treatment groups to lesional results, p value indicated.

IL-8, which are considered to be immediate response genes to induction by TNF, are strongly suppressed at all analysis time points, and the suppression appears to be maximal by 1 mo of treatment. In contrast, most other gene products show more gradual reductions and, in general, they are most strongly suppressed after 6 mo of continuous etanercept treatment. At the 1-mo time point, the degree to which late genes were suppressed by etanercept was highly variable in different patients, but the suppression was much more consistent at later time points (the p values reflect this response trend).

The relationship between individual gene expression at 1 mo of etanercept treatment and psoriatic disease phenotype (as measured by the RS) is demonstrated in Table I (r score). This time point was chosen because this is when the largest range of responses were evident. Psoriasis disease improvement at 1 mo was highly related to IL-1, MMP-12, and several type 1 pathway products (STAT-1, IL-23, MIG, IL-8, and iNOS). However, changes in gene expression should be considered in the context of reduced numbers of infiltrating leukocytes during the analysis period. The correlations between disease improvement and iNOS mRNA levels ($r = 0.83$; Table I) and $CD11c^+$ DCs ($r = 0.70$; Fig. 3) are interrelated events, because iNOS production is restricted to this cell subset (see analysis below). Similarly, reductions in T cells in tissue could explain part of the reduction in gene expression linked to T cells, e.g., IFN-γ or granzyme B mRNAs. Additionally, total plaque neutrophil counts decreased from a mean of 485 ± 368 in pretreatment plaques to 170 ± 343 ($p = 0.03$) after only 1 mo of etanercept treatment (as quantitated by neutrophil elastase). Reductions in total neutrophil counts correlated with decreased IL-8 gene expression (data not shown). Even so, the suppression of lineage-associated inflammatory genes occurs more quickly and to a larger extent than overall reductions in associated leukocyte subsets. The observed decreases in IL-8, IP-10, and MIP-3α mRNA expression may account for decreased infiltration of neutrophils, T cells, and DCs, respectively.

Because inflammatory gene sets may be additive or interactive for producing pathogenic inflammation, we also used a novel method to assess which combination of genes was most highly

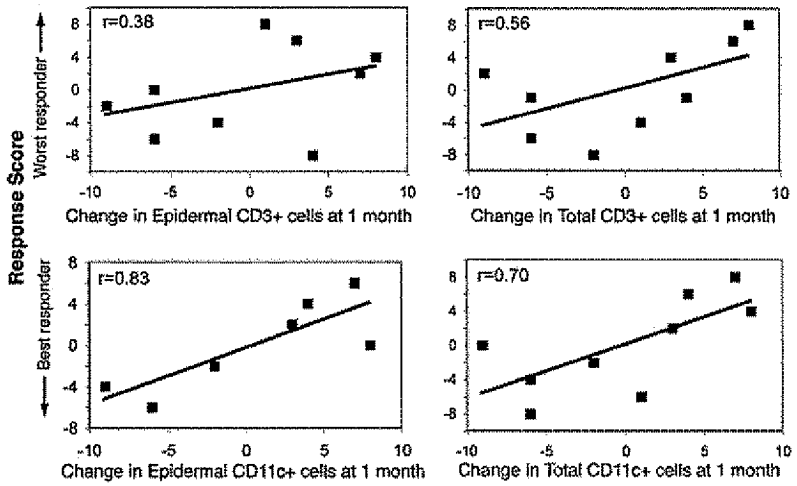

FIGURE 3. Correlation of RS with decreased numbers of $CD3^+$ cells or $CD11c^+$ cells at 1 mo of treatment. RS describes psoriasis disease activity across the range of complete disease resolution to highly active disease present, and stratifies patient responses from best (lowest score) to worst (highest score). This score is then correlated with change in epidermal and total $CD3^+$ and $CD11c^+$ cells at 1 mo (positive value is least change/reduction, negative value is highest change/reduction), with r value for each comparison indicated.

correlated with the RS at different analysis time points (27). At 1 mo, there was a high correlation ($r = 0.91, p < 0.001$) between the combined expression of IL-1, STAT-1, and IL-23/IL-12 p40 mRNAs, as illustrated in the bottom right panel of Fig. 4. However, the final degree of disease improvement seen at 6 mo was associated with a different set of inflammatory gene products, with the combined expression of IFN-γ, granzyme B, and IL-19 mRNAs most correlated ($r = 0.93$) with the RS (data not shown). Thus, the disease-resolution response to etanercept is a rapid and complete reduction in IL-1 and IL-8 (immediate/early genes), followed by progressive reductions in many other inflammation-related genes, and finally somewhat slower reductions in infiltrating myeloid cells and T lymphocytes.

Etanercept causes a decrease in iNOS protein in DCs

In general, expression of inflammatory genes was decreased to a greater extent than infiltrating DCs or T cells. This was particularly true for the earlier time points and suggests that etanercept reduces expression of inflammatory genes produced by infiltrating leukocytes, rather than just reducing trafficking of leukocytes and constitutive gene products. Recently, we have determined that iNOS-producing myeloid ($CD11c^+$) DCs are tremendously increased in psoriasis lesions (29). Because iNOS mRNA is jointly regulated by NFκB and STAT-1 (TNF- and IFN-induced transcription factors, respectively), we examined the production of iNOS protein in $CD11c^+$ cells in lesions using two-color immunofluorescence microscopy (Fig. 5). We

Table I. *Functional significance of genes evaluated during etanercept treatment of psoriasis patients, and correlation of mRNA level and RS at 1 mo*

Gene	Functional Significance	Correlation (r) with RS at 1 mo
Early TNF-induced genes		
IL-1β	Inflammatory cytokine	0.70
IL-8	Inflammatory cytokine for neutrophils, also product of type 1 pathway	0.77
MIP-3α (CCL20)	Most potent chemokine for DC attraction, inflammatory cytokine, also may be produced by IL-1 stimulation	0.65
IL-6	Inflammatory cytokine	0.06
Type 1 pathway genes (induced or regulated by IFN-γ)		
IL-23	Proximal type 1 pathway gene, induces IFN-γ	0.72
STAT-1	Transcription factor induced by IFN-γ, central factor of type 1 pathway	0.69
MIG	Chemokine regulated mainly by IFN-γ, inflammatory cytokine	0.78
iNOS	Coregulated by TNF/IFN-γ, produces NO, inflammatory mediator	0.83
IP-10 (CXCL10)	Chemokine regulated by TNF/IFN-γ, chemotaxis T cells, inflammatory cytokine	0.79
IL-8	Type 1 pathway product, chemokine for neutrophils	0.77
Myeloid genes		
iNOS	Produces NO, inflammatory mediator	0.83
IL-19	Product of activated monocytes/myeloid cells, inflammatory cytokine	0.72
MMP-12	Product activated macrophages/myeloid cells, metalloelastase	0.74
IL-23p19	Defining IL-23 subunit, inducer of IFN-γ, inflammatory cytokine, proximal type 1 pathway gene	0.72
IL-23p40	Shared subunit with IL-12 and IL-23, inflammatory cytokine	0.14
CD83	Marker of mature/activated DCs	0.31
Lymphocyte genes		
Granzyme B	Product of activated $CD8^+$ T cells, inflammatory mediator	0.86
IFN-γ	Product of stimulated type 1 T cell, proximal type 1 pathway gene	0.20

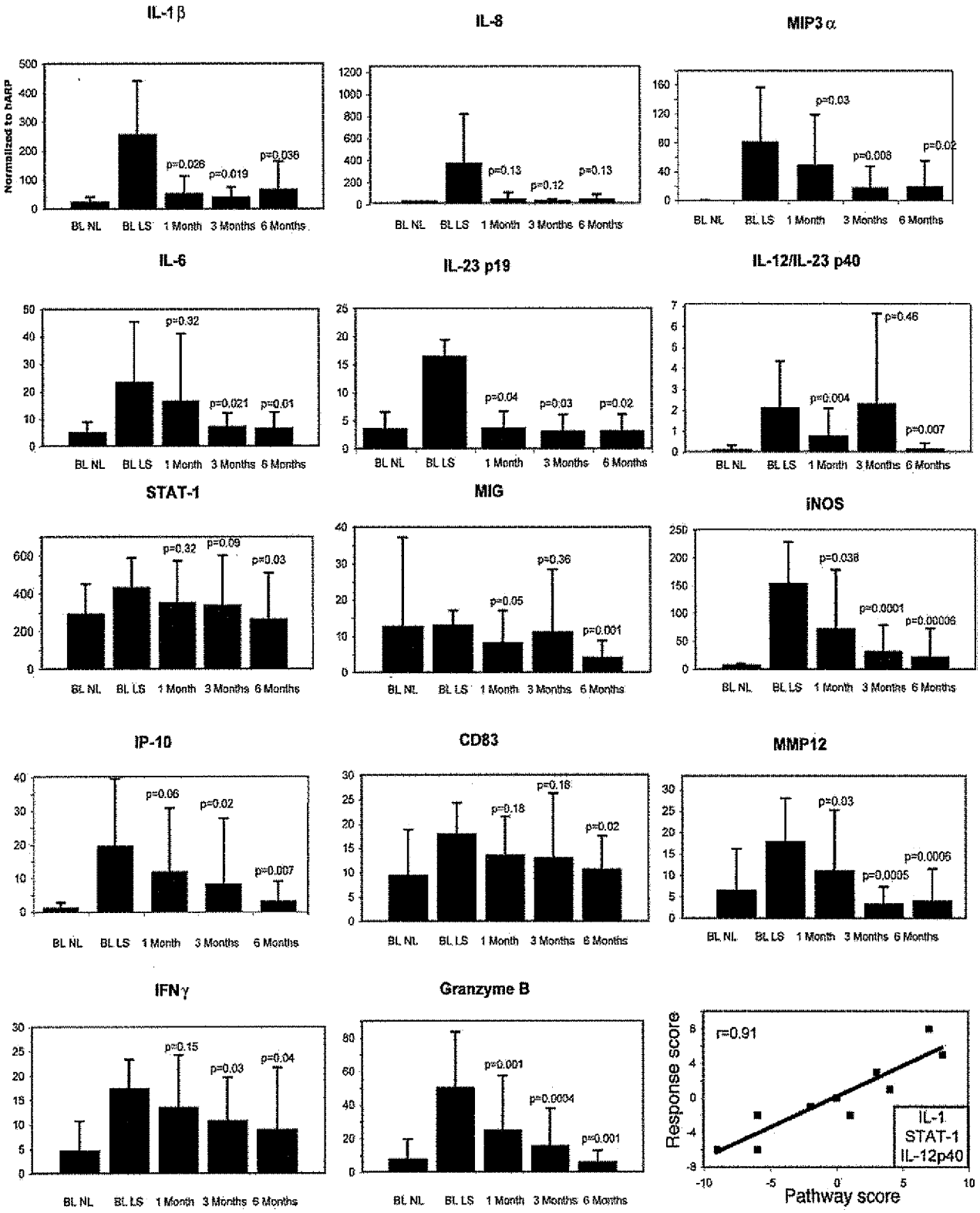

FIGURE 4. Reduced inflammatory gene expression in response to etanercept treatment. Results of RT-PCR for a panel of genes increased in psoriasis and the effect of etanercept treatment on their expression (normalized to HARP) at baseline noniesional (BL NL), baseline lesional (BL LS), and after 1, 3, and 6 mo of treatment. Comparison of results in treatment groups to lesional results, p value indicated. Bottom right panel indicates the correlation at 1 mo between RS and pathway score (comprising change in expression of a combination of IL-1, STAT-1, IL-12p40), with r value indicated.

consistently observed that before etanercept treatment, there were abundant $CD11c^+/iNOS^+$ cells. However, in etanercept-treated biopsies, there were numerous $CD11c^+$ cells with little or no detectable iNOS protein expression.

Because iNOS expression by DCs can be viewed as a TNF-mediated activation-related response, we also studied expression of other activation or maturation-related DC products. The IL-23p19 mRNA, which is induced in monocyte-derived DCs in response to a

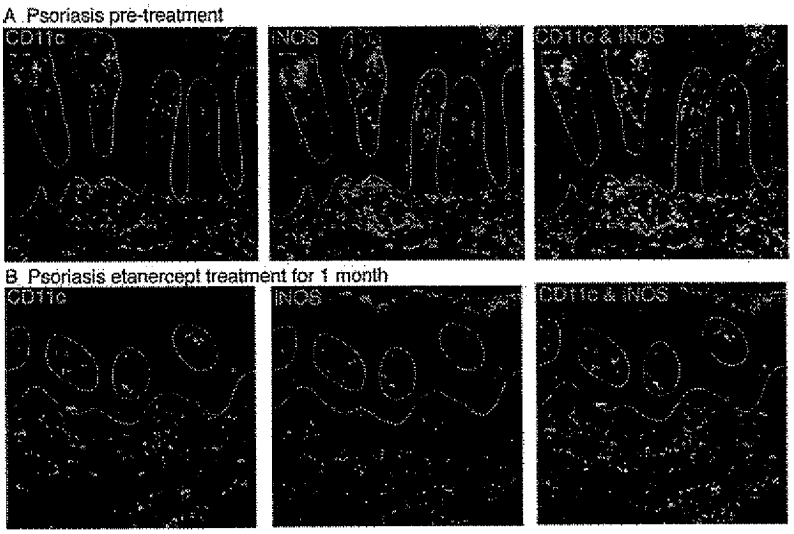

FIGURE 5. Reduction in iNOS protein expression in CD11c$^+$ cells after 1 mo of etanercept treatment. *A*, Psoriasis lesional skin showing numerous epidermal and dermal CD11c, iNOS double-positive cells (yellow cells in right panel). *B*, Substantial reduction with etanercept treatment for 1 mo.

TNF-containing maturation stimulus, was significantly down-regulated in 3- and 6-mo biopsies (Fig. 4). Another marker of mature/activated DCs is CD83. We observed virtual elimination of CD83 immunoreactivity in several etanercept-treated cases (data not shown) and in agreement with reduced CD83 mRNA expression by 3 mo (Fig. 4). Limited availability of biopsy sections prevented quantitative analysis of CD83 expression in etanercept-treated lesions from all patients; thus a formal correlation between response and CD83 protein expression could not be attained.

Discussion

Past studies of therapeutic mechanism(s) with TNF antagonists have been conducted in patients with rheumatoid arthritis and psoriatic arthritis. Several studies support the general view that TNF antagonists reduce expression of vascular adhesion molecules (selectins, VCAM, ICAM-1) with attendant reductions in trafficking of leukocytes to inflamed synovium. However, much of this analysis has been focused on circulating cytokines, soluble fragments of cell adhesion molecules, and properties of peripheral leukocytes, because access to the affected tissue is difficult (38–46). Analyses conducted on inflamed synovium have shown reduced infiltration by CD68$^+$ myeloid cells (most likely tissue macrophages), but reductions in infiltrating T cells have been measured less consistently (47, 48). Reduced expression of mRNA for several cytokines that regulate angiogenesis has been measured in synovial tissue after infliximab treatment (48), but comparable data for inflammatory cytokines do not exist and a time course of cellular/molecular inflammatory events is difficult to construct.

In contrast, the accessibility of diseased skin tissue in psoriasis has made it possible to study sequential alterations in inflammatory leukocytes and numerous inflammation-associated gene products during etanercept treatment. Although it may be predictable that inflammatory genes and infiltrating cells are decreased by this therapy, it is important to perform a detailed characterization and time course of these events to develop insights into such issues as why patients respond, the molecular and cellular mechanisms of action, and developing new applications for biological therapies.

We have developed methods to quantify the extent of disease burden via cellular and genomic measures of epidermal hyperplasia (the response score or RS) and methods to quantify infiltrating leukocytes and numerous inflammation-associated genes within a single biopsy specimen. Our analysis of the therapeutic mechanism of etanercept has also been greatly aided by a series of recent genomic studies of psoriasis vulgaris that point to a series of inflammatory genes (type 1 genes), which can act in a sequential manner or a cascade to produce numerous cellular features of this disease (36, 49). The view of therapeutic action of etanercept obtained in this study is much more detailed than any previous study of a TNF inhibitor in human disease, and it is conceptually different from most models of TNF inhibition in arthritis.

The genomic approach to study inflammatory gene expression after etanercept treatment is based on the idea that genes induced by TNF or LT will be suppressed by cytokine blockade. However, expression of TNF or LT mRNA in producing cells may not be affected by blocking the protein product, because they are likely to regulated by other stimuli. Hence, we have not concentrated on these gene products (TNF and LT) in the downstream analysis of inflammatory gene expression.

In this study, the most consistent cellular effects of etanercept were 1) reductions in keratinocyte hyperplasia (reflected in epidermal thickness measures, K16 expression, and the integrated RS) and 2) reductions in CD11c$^+$ myeloid cells, which are best classified as myeloid DCs (26). Virtually all CD11c$^+$ DCs synthesize iNOS at high levels, and we found that reduced expression of iNOS mRNA as a single gene product was highly correlated with etanercept-induced therapeutic improvement (Table I), whereas expression of iNOS protein was clearly reduced in CD11c$^+$ cells (Fig. 5). The enzyme iNOS produces NO from arginine and NO is a molecule with both cell-damaging properties and the ability to dilate small blood vessels. In psoriasis, NO may be a trigger for keratinocyte hyperplasia and a factor accounting for skin erythema, through its vasoactive effects (50, 51). Up-regulation of iNOS mRNA in DCs may also be a general sentinel of activation occurring in this cell type. Past studies in mice have found critical roles of TNF and LT in the generation of DCs (52–55), so a central effect of TNF/LT blockade in psoriatic lesions may be related to blocked in situ differentiation of these cells from circulating or resident precursors. The reduced expression of IL-23, previously traced to myeloid DCs in psoriasis lesions (56) and reduced expression of CD83 (a marker of mature DCs in humans), also suggest a major impact of etanercept on DCs in psoriasis. Changes in T cell infiltrates and reduced expression of IFN-γ and granzyme B may all be secondary to reduced stimulation of T cells in situ by activated DCs. We note, however, that reduced synthesis of IFN-γ by T cells might also be directly attributed to TNF inhibition (57). Gradual reductions in T cells and DCs in psoriasis

skin lesions may also be produced by reductions in IP-10 and MIP-3α, respectively (30, 58). Rapid reductions in neutrophils may be produced by reductions in IL-8 (59). ICAM-1 expression by dermal blood vessels did not appear to be reduced by etanercept (data not shown). Accordingly, a reduced chemokine stimulus for cell migration into skin seems more likely as a mechanism to explain decreased migration of leukocytes into the dermis. In contrast, ICAM-1 expression by keratinocytes is down-regulated during etanercept treatment; thus, migration of leukocytes from the dermis into the epidermis could be blocked by direct modulation of adhesion molecules (60, 61).

The direct activation of epidermal keratinocytes by TNF or LT may also be an important pathogenic mechanism in psoriasis. A recent study using gene arrays has identified >70 genes that are up-regulated in human keratinocytes after TNF exposure (1). A large number of TNF-induced genes encode chemokines, cytokines, and other proinflammatory molecules that would be expected to enhance leukocyte recruitment into the skin and subsequent cellular activation. IL-1β, IL-8, and IP-10 were identified as TNF-regulated products in keratinocytes using gene arrays, and all of these genes were strongly suppressed in psoriasis lesions during etanercept treatment. ICAM-1 mRNA was induced in keratinocytes after TNF treatment, and our data show decreased expression of ICAM-1 protein in keratinocytes after etanercept treatment. TNF also induces cytokines that stimulate proliferation of resident cells in the skin. TGF-α, nerve growth factor, endothelial cell growth factor, and vascular endothelial cell growth factor are increased in keratinocytes after TNF treatment. These factors were not studied in cutaneous lesions during etanercept treatment, but several angiogenic cytokines are decreased in synovium from psoriatic arthritis after TNF blockade. Accordingly, it seems likely that etanercept exerts major therapeutic actions in psoriasis skin lesions by down-regulating expression of proinflammatory and proproliferative genes that are induced in keratinocytes (as well as other skin-resident cell types) by local synthesis of TNF.

The inflammatory response to TNF could be self-sustaining, because activated DCs are a major source of TNF in psoriasis lesions (18) and because TNF mRNA is induced in keratinocytes after TNF exposure (1). Treatment with etanercept could provide the means to break this self-amplifying inflammatory cascade, but etanercept produces relatively slow down-regulation of some proinflammatory genes in psoriasis lesions. The progressive down-regulation of genes like IP-10 or iNOS during 6 mo of treatment with etanercept contrasts sharply with the rapid induction of these genes in cultured cells after treatment with TNF (1) and also with rapid cellular responses to TNF in sepsis. The most likely explanation for this apparent discrepancy is that proinflammatory genes like IP-10 and iNOS are controlled by multiple transcription factors induced by different classes of inflammatory cytokines (62). Hence, in chronic inflammatory diseases, TNF might broadly tune levels of proinflammatory gene expression, rather than act as a strict on/off switch for inflammatory responses. The expression of TNF and/or LT in cutaneous inflammatory sites may also promote in situ activation/maturation of myeloid DCs, such that therapeutic inhibition of these cytokines gradually collapses organized T/DC infiltrates that sustain inflammation in the skin (36). Accordingly, TNF/LT cytokines can effectively bridge innate and acquired immune responses by inducing early inflammatory genes and also supporting the development of other cellular and cytokine networks that lead to long range enhancement of T cell-mediated responses.

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References

1. Banno, T., A. Gazel, and M. Blumenberg, 2004. Effects of tumor necrosis factor-α (TNFα) in epidermal keratinocytes revealed using global transcriptional profiling. *J. Biol. Chem.* 279: 32633–32642.
2. Norris, D. A. 1990. Cytokine modulation of adhesion molecules in the regulation of immunologic cytotoxicity of epidermal targets. *J. Invest. Dermatol.* 95: 111S–120S.
3. Old, L. J. 1985. Tumor necrosis factor (TNF). *Science* 230: 630–632.
4. Zhou, A., S. Scoggin, R. Gaynor, and N. S. Williams. 2003. Identification of NFκB-regulated genes induced by TNFα utilizing expression profiling and RNA interference. *Oncogene* 22: 2054–2064.
5. Mansbridge, J. N., and A. M. Knapp. 1987. Changes in keratinocyte maturation during wound healing. *J. Invest. Dermatol.* 89: 253–263.
6. Gottlieb, A. B., R. M. Grossman, L. Khandke, D. M. Carter, P. B. Sehgal, S. M. Fu, A. Granelli-Piperno, M. Rivas, L. Barazani, and J. G. Krueger. 1992. Studies of the effect of cyclosporine in psoriasis in vivo: combined effects on activated T lymphocytes and epidermal regenerative maturation. *J. Invest. Dermatol.* 98: 302–309.
7. Gottlieb, A. B., B. Lifshitz, S. M. Fu, L. Staiano-Coico, C. Y. Wang, and D. M. Carter. 1986. Expression of HLA-DR molecules by keratinocytes and presence of Langerhans cells in the dermal infiltrate of active psoriatic plaques. *J. Exp. Med.* 164: 1013–1028.
8. Gottlieb, A. B., and J. G. Krueger. 1990. HLA region genes and immune activation in the pathogenesis of psoriasis. *Arch. Dermatol.* 126: 1083–1091.
9. Gottlieb, A. B., J. G. Krueger, L. Khandke, R. M. Grossman, J. Krane, and D. M. Carter. 1991. Role of T cell activation in the pathogenesis of psoriasis. *Ann. NY Acad. Sci.* 636: 377–379.
10. Gottlieb, S. L., P. Gilleaudeau, R. Johnson, L. Estes, T. G. Woodworth, A. B. Gottlieb, and J. G. Krueger. 1995. Response of psoriasis to a lymphocyte-selective toxin (DAB389IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. *Nat. Med.* 1: 442–447.
11. Krueger, J. 2002. The immunologic basis for the treatment of psoriasis with new biologic agents. *J. Am. Acad. Dermatol.* 46: 1–26.
12. Nestle, F., L. Turka, and B. Nickoloff. 1994. Characterization of dermal dendritic cells in psoriasis: autostimulation of T lymphocytes and induction of Th1 type cytokines. *J. Clin. Invest.* 94: 202–209.
13. Nickoloff, B. J., and C. E. M. Griffiths. 1990. Lymphocyte trafficking in psoriasis: a new perspective emphasizing the dermal dendrocyte with active dermal recruitment mediated via endothelial cells followed by intra-epidermal T-cell activation. *J. Invest. Dermatol.* 95: 35S–37S.
14. Baker, B. S., A. F. Swain, C. E. M. Griffiths, J. N. Leonard, L. Fry, and H. Valdimarsson. 1985. Epidermal T lymphocytes and dendritic cells in chronic plaque psoriasis; the effects of PUVA treatment. *Clin. Exp. Immunol.* 61: 526–534.
15. Bos, J. D., I. D. VanGarderen, S. R. Krieg, and L. W. Poulter. 1986. Different in situ distribution patterns of dendritic cells haveing Langerhans (T6) and interdigitating (RFD^+) cell immunophenotype in psoriasis, atopic dermatitis, and other inflammatory dermatoses. *J. Invest. Dermatol.* 87: 358–361.
16. Wrone-Smith, T., and B. J. Nickoloff. 1996. Dermal injection of immunocytes induces psoriasis. *J. Clin. Invest.* 98: 1878–1887.
17. Gilhar, A., M. David, Y. Ullmann, T. Berkutski, and R. S. Kalish. 1997. T-lymphocyte dependence of psoriatic pathology in human psoriatic skin grafted to SCID mice. *J. Invest. Dermatol.* 109: 283–288.
18. Boyman, O., H. P. Hefti, C. Conrad, B. Nickoloff, M. Suter, and F. O. Nestle. 2004. Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor α. *J. Exp. Med.* 199: 731–736.
19. Chaudhari, U., P. Romano, L. D. Mulcahy, L. T. Dooley, D. G. Baker, and A. B. Gottlieb. 2001. Efficacy and safety of infliximab monotherapy for plaque-type psoriasis: a randomised trial. *Lancet* 357: 1842–1847.
20. Gottlieb, A. B., S. Masud, R. Ramamurthi, A. Abdulshani, P. Romano, U. Chaudhari, L. T. Dooley, A. A. Fasanmade, and C. Wagner. 2003. Pharmacodynamic and pharmacokinetic response to anti-tumor necrosis factor-α monoclonal antibody (infliximab) treatment of moderate to severe psoriasis vulgaris. *J. Am. Acad. Dermatol.* 48: 68–75.
21. Gottlieb, A. B. 2003. Clinical research helps elucidate the role of tumor necrosis factor-α (TNF-α) in the pathogenesis of T1 mediated immune disorders: use of targeted immunotherapeutics as pathogenic probes. *Lupus* 12: 192–194.
22. Gottlieb, A. B., R. T. Matheson, N. Low, G. G. Krueger, S. Kang, B. S. Goffe, A. A. Gaspari, M. Ling, G. D. Weinstein, A. Nayak, K. B. Gordon, and R. Zitnik. 2003. A randomized trial of etanercept as monotherapy for psoriasis. *Arch. Dermatol.* 139: 1627–1632.
23. Gottlieb, A. B. 2004. Etanercept for the treatment of psoriasis and psoriatic arthritis. *Dermatol. Ther.* 17: 401–408.
24. Leonardi, C. L., J. L. Powers, R. T. Matheson, B. Goffe, R. Zitnik, A. Wang, and A. B. Gottlieb. 2003. Etanercept as monotherapy in patients with psoriasis. *N. Engl. J. Med.* 349: 2014–2022.
25. Frederiksson, T., and U. Pettersson. 1978. Severe psoriasis oral therapy with a new retinoid. *Dermatologica* 157: 238–244.

26. Chamian, F., M. A. Lowes, S.-L. Lin, E. Lee, T. Kijuchi, P. Gilleaudeau, M. Sullivan-Whalen, I. Cardinale, A. Khatcherian, I. Novitskaya, K. M. Wittkowski, and J. G. Krueger. 2005. Alefacept reduces infiltrating T cells, activated dendritic cells and inflammatory genes in psoriasis vulgaris. *Proc. Natl. Acad. Soc. USA* 102: 2075–2080.
27. Wittkowski, K. M., E. Lee, R. Nussbaum, F. Chamian., and J. G. Krueger. 2004. Combining several ordinal measures in clinical studies. *Stat. Med.* 23: 1579–1592.
28. Wollenberg, A., M. Wagner, S. Gunther, A. Towarowski, E. Tuma, M. Moderer, S. Rothenfusser, S. Wetzel, S. Endres, and G. Hartmann. 2002. Plasmacytoid dendritic cells: a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. *J. Invest. Dermatol.* 119: 1096–1102.
29. Chamian, F., S. Lin, I. Novitskaya, H. Carbonaro, I. Cardinale, T. Kikuchi, P. Gilleaudeau, K. Wittkowski, K. Papp, M. R. Garovoy, W. Dummer, and J. G. Krueger. 2004. Presence of "inflammatory" dendritic cells in psoriasis vulgaris lesions and modulation by efalizumab (anti-CD11a). *J. Invest. Dermatol.* 122: A41.
30. Gottlieb, A. B., A. D. Luster, D. N. Posnett, and D. M. Carter. 1988. Detection of a γ-interferon-induced protein (IP-10) in psoriatic plaques. *J. Exp. Med.* 168: 941–948.
31. Grossman, R. M., J. Krueger, D. Yourish, A. Granelli-Piperno, D. P. Murphy, L. T. May, T. S. Kupper, P. B. Sehgal, and A. B. Gottlieb. 1989. Interleukin-6 (IL-6) is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. *Proc. Natl. Acad. Sci. USA* 86: 6367–6371.
32. Austin, L., M. Ozawa, T. Kikuchi, I. Walters, and J. Krueger. 1999. The majority of epidermal T cells in psoriasis vulgaris lesions can produce type 1 cytokines, interferon-γ, interleukin-2, and tumor necrosis factor-α, defining TC1 (cytotoxic T lymphocyte) and Th1 effector populations: a type 1 differentiation bias is also measured in circulating blood T cells in psoriatic patients. *J. Invest. Dermatol.* 113: 752–759.
33. Lee, E., W. Trepicchio, J. Oestreicher, D. Pittman, F. Wang, F. Chamian, M. Dhodapkar, and J. G. Krueger. 2004. Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *J. Exp. Med.* 199: 125–130.
34. Oestreicher, J. L., I. B Walters, T. Kikuchi, P. Gilleaudeau, J. Surette, U. Schwertschlag, A. J. Dorner, J. G. Krueger, and W. L. Trepicchio. 2001. Molecular classification of psoriasis disease-associated genes through pharmacodynamic expression profiling. *Pharmacogenomics J.* 1: 272–287.
35. Suomela, S., A. L. Kariniemi, E. Snellman, and U. Saarialho-Kere. 2003. Metalloelastase (MMP-12) and 92-kDa gelatinase (MMP-9) as well as their inhibitors, TIMP-1 and -3, are expressed in psoriatic lesions. *Exp. Dermatol.* 10: 175–183.
36. Lew, W., A. M. Bowcock, and J.G. Krueger. 2004. Psoriasis vulgaris: cutaneous lymphoid tissue supports T-cell activation and "type 1" inflammatory gene expression. *Trends Immunol.* 25: 295–305.
37. Lew, W., E. Lee, and J. G. Krueger. 2004. Psoriasis genomics: analysis of proinflammatory (type 1) gene expression in large plaque (Western) and small plaque (Asian) psoriasis vulgaris. *Br. J. Dermatol.* 150: 668–676.
38. Paleolog, E. M., M. Hunt, M. J. Elliott, M. Feldmann, R. N. Maini, and J. N. Woody. 1996. Deactivation of vascular endothelium by monoclonal anti-tumor necrosis factor-α antibody in rheumatoid arthritis. *Arthritis Rheum.* 39: 1082–1091.
39. Schuerwegh, A. J., J. F. Van Offel, W. J. Stevens, C. H. Bridts, and L. S. De Clerck. 2003. Influence of therapy with chimeric monoclonal tumour necrosis factor-α antibodies on intracellular cytokine profiles of T lymphocytes and monocytes in rheumatoid arthritis patients. *Rheumatology* 42: 541–548.
40. Schotte, H., B. Schluter, P. Willeke, E. Mickholz, M. Schorat, V. Domschke, and M. Gaubitz. 2004. Long-term treatment with etanercept significantly reduces the number of proinflammatory cytokine-secreting peripheral blood mononuclear cells in patients with rheumatoid arthritis. *Rheumatology* 43: 960–964.
41. Catrina, A. I., J. Lampa, S. Ernestam, E. af Klint, J. Bratt, L. Klareskog, and A. K. Ulfgren. 2002. Anti-tumour necrosis factor (TNF)-α therapy (etanercept) down-regulates serum matrix metalloproteinase (MMP)-3 and MMP-1 in rheumatoid arthritis. *Rheumatology* 41: 484–489.
42. Brennan, F. M., K. A. Browne, P. A. Green, J.-M. Jaspar, R. N. Maini, and M. Feldmann. 1997. Reduction of serum matrix metalloproteinase 1 and matrix metalloproteinase 3 in rheumatoid arthritis patients following anti-tumour necrosis factor-α (cA2) therapy. *Br. J. Rheum.* 36: 643–650.
43. Klimiuk, P., S. Sierakowski, I. Domyslawaska, and J. Chwiecko. 2004. Effect of repeated infliximab therapy on serum matrix metalloproteinases and tissue inhibitors of metalloproteinases in patients with rheumatoid arthritis. *J. Rheumatol.* 31: 238–240.
44. Drynda, S., C. Kuhne, and J. Kekow. 2002. Soluble tumour necrosis factor receptor treatment does not affect raised transforming growth factor β levels in rheumatoid arthritis. *Ann. Rheum. Dis.* 61: 254–256.
45. Tokayer, A., S. Carsons, B. Chokshi, and F. Santiago-Schwarz. 2004. High levels of interleukin 13 in rheumatoid arthritis sera are modulated by tumor necrosis factor antagonist therapy: associated with dendritic cell growth activity. *J. Rheumatol.* 29: 454–461.
46. Agnholt, J., J. F. Dahlerup, and K. Kaltoft. 2003. The effect of etanercept and infliximab on the production of tumour necrosis factor α, interferon-γ and GM-CSF in in vivo activated intestinal T lymphocyte cultures. *Cytokines* 23: 76–85.
47. Tak, P.-P., P. C. Taylor, F. C. Breedveld, T. Smeets, M. R. Daha, P. M. Kluin, A. E. Meinders, and R. N. Maini. 1996. Decrease in cellularity and expression of adhesion molecules by anti-tumor necrosis factor α monoclonal antibody treatment in patients with rheumatoid arthritis. *Arthritis Rheum.* 39: 1077–1081.
48. Canete, J. D., J. L. Pablos, R. Sanmarti, C. Malloffre, S. Marsal, J. Maymo, J. Gratacos, J. Mezquita, C. Mezquita, and M. C. Cid. 2004. Antiangiogenic effects of anti-tumor necrosis factor α therapy with infliximab in psoriatic arthritis. *Arthritis. Rheum.* 50: 1636–1641.
49. Zhou, X., J. G. Krueger, M.-C. J. Kao, E. Lee, F. Du, A. Menter, M. D., W. H. Wong, and A. M. Bowcock. 2003. Novel mechanisms of T-cell and dendritic cell activation revealed by profiling of psoriasis on the 63,100-element oligonucleotide array. *Physiol. Genomics* 13: 69–78.
50. Bogdan, C. 2001. Nitric oxide and the immune response. *Nat. Immunol.* 2: 907–916.
51. Morhenn, V. B. 1997. Langerhans cells may trigger the psoriatic disease process via production of nitric oxide. *Immunol. Today* 18: 433–436.
52. van Lieshout, A. W. T., P. Barrerera, R. L. Smeets, G. J. Pesman, P. L. C. M. van Riel, W. B. vanden Berg, and T. R. D. J. Radstake. 2005. Inhibition of TNF-α during maturation of dendritic cells results in the development of semi-mature DC: a potential mechanism for the beneficial effects of TNFα blockade in rheumatoid arthritis. *Ann. Rheum. Dis.* 64: 408–414.
53. Ritter, U., A. Meissner, J. Ott, and H. Korner. 2003. Analysis of the maturation process of dendritic cells deficient for TNF and lymphotoxin-α reveals an essential role for TNF. *J. Leukocyte Biol.* 74: 216–222.
54. Abe, K., F. O. Yarovinsky, T. Murakami, A. N. Shakhov, A. V. Tumanov, D. Ito, L. N. Drutskaya, K. Pfeffer, D. V. Kuprash, K. L. Kornschlies, and S. A. Nedospasov. 2003. Distinct contributions of TNF and LT cytokines to the development of dendritic cells in vitro and their recruitment in vivo. *Blood* 101: 1477–1483.
55. Fukaya, H., W. Xiao, K. Inaba, Y. Suzuki, M. Hirokawa, Y. Hawabata, A. Komatsuda, T. Endo, H. Kishimotok, G. Tadada, and K. Sawada. 2004. Co-development of dendritic cells along with erythroid differentiation from human $CD34^+$ cells by tumor necrosis factor α. *Exp. Hematol.* 32: 450–460.
56. Gottlieb, A. B., R. Evans, S. Li, L. T. Dooley, C. Guzzo, A. D. Baker, M. Bala, C. W. Marano, and A. Menter. 2004. Infliximab induction therapy for patients with severe plaque-type psoriasis: a randomized, double-blind, placebo-controlled trial. *J. Am. Acad. Dermatol.* 51: 534–542.
57. Agnholt, J., and K. Kaltoft. 2001. Infliximab downregulates interferon-γ production in activated gut T-lymphocytes from patients with Crohn's disease. *Cytokines* 15: 212–222.
58. Homey, B., M. Dieu-Nosjean, A. Wiesenborn, C. Massacrier, J. Pin, E. Oldham, D. Catron, M. Buchanan, A. Muller, R. de Waal Malfyt, G. Deng, R. Orozco, T. Ruzicka, P. Lehmann, S. Lebecque, C. Caux, and A. Zlotnik. 2000. Up-regulation of macrophage inflammatory protein-3 α/CCL20 and CC chemokine receptor 6 in psoriasis. *J. Immunol.* 164: 6621–6632.
59. Barker, J. N., M. L. Jones, R. S. Mitra, E. Crockett-Torabe, J. C. Fantone, S. L. Kunkel, J. S. Warren, V. M. Dixit, and B. J. Nickoloff. 1991. Modulation of keratinocyte-derived interleukin-8 which is chemotactic for neutrophils and T lymphocytes. *Am. J. Pathol.* 139: 869–876.
60. Rothlein, R., M. Czajkowski, M. M. O'Neill, S. D. Marlin, E. Mainolfi, and V. J. Merluzzi. 1988. Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines: regulation by pharmacologic agents and neutralizing antibodies. *J. Immunol.* 141: 1665–1669.
61. Singer, K. H., D. T. Tuck, H. A. Sampson, and R. P. Hall. 1989. Epidermal keratinocytes express the adhesion molecule intercellular adhesion molecule-1 in inflammatory dermatoses. *J. Invest. Dermatol.* 92: 746–750.
62. Pine, R. 1997. Convergence of TNFα and IFNγ signalling pathways through synergistic induction of IRF-1/ISGF-2 is mediated by a composite GAS/κB promoter element. *Nucleic Acids Res.* 25: 4346–4354.

Protein Expression of TNF-α in Psoriatic Skin Is Regulated at a Posttranscriptional Level by MAPK-Activated Protein Kinase 2[1]

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Alterations in specific signal transduction pathways may explain the increased expression of proinflammatory cytokines seen in inflammatory diseases such as psoriasis. We reveal increased TNF-α protein expression, but similar TNF-α mRNA levels, in lesional compared with nonlesional psoriatic skin, demonstrating for the first time that TNF-α expression in lesional psoriatic skin is regulated posttranscriptionally. Increased levels of activated MAPK-activated protein kinase 2 (MK2) together with increased MK2 kinase activity were found in lesional compared with nonlesional psoriatic skin. Immunohistochemical analysis showed that activated MK2 was located in the basal layers of the psoriatic epidermis, whereas no positive staining was seen in nonlesional psoriatic skin. In vitro experiments demonstrated that both anisomycin and IL-1β caused a significant activation of p38 MAPK and MK2 in cultured normal human keratinocytes. In addition, TNF-α protein levels were significantly up-regulated in keratinocytes stimulated with anisomycin or IL-1β. This increase in TNF-α protein expression was completely blocked by the p38 inhibitor, SB202190. Transfection of cultured keratinocytes with MK2-specific small interfering RNA led to a significant decrease in MK2 expression and a subsequent significant reduction in the protein expression of the proinflammatory cytokines TNF-α, IL-6, and IL-8, whereas no change in the expression of the anti-inflammatory cytokine IL-10 was seen. This is the first time that MK2 expression and activity have been investigated in an inflammatory disease such as psoriasis. The results strongly suggest that increased activation of MK2 is responsible for the elevated and posttranscriptionally regulated TNF-α protein expression in psoriatic skin, making MK2 a potential target in the treatment of psoriasis. *The Journal of Immunology,* **2006, 176: 1431–1438.**

Psoriasis is an immune-mediated inflammatory skin disorder characterized by skin-infiltrating lymphocytes causing hyperproliferation and abnormal differentiation of the keratinocytes (1, 2). The initiation and persistence of the characteristic inflammatory processes in psoriasis seem to be triggered by a cytokine pattern belonging to the Th1 type. This pattern consists of signaling molecules such as TNF-α, IFN-γ, IL-1, IL-2, IL-3, IL-6, IL-8, epidermal growth factor, and TGF-α (3, 4). Because of the strong antipsoriatic activity of TNF-α antagonists, the increased TNF-α expression is of particular interest. However, the intracellular signaling pathways implicated in this altered cytokine pattern remain to be determined.

The p38 MAPK is activated by cellular stress, such as anisomycin, heat shock, H_2O_2, and UV radiation; by several proinflammatory cytokines, including TNF-α and IL-1; and LPS (5, 6). There are four p38 MAPK isoforms: the α and β isoforms, which are 75% homologous, and the γ and δ isoforms, which are more distant relatives (7). We have recently demonstrated activation of p38α, -β, and -δ in lesional psoriatic skin compared with nonlesional psoriatic skin, whereas p38γ is not expressed in human epidermis (8). A major function of p38α and p38β in inflammation is regulation of the expression of inflammatory cytokines (9). The mechanism by which p38 MAPK mediates its regulatory effects is through several p38 MAPK downstream kinases, including the MAPK-activated protein kinase 2 (MAPKAP kinase 2 (MK2)[7]) (10–12).

MK2 is a serine/threonine kinase that is phosphorylated and activated by the p38α and p38β MAPKs (13). In its inactive form, MK2 is located in the nucleus. Upon activation by the p38α and -β MAPKs, MK2 rapidly translocates from the nucleus to the cytoplasm and also cotransports p38 MAPK back to the cytoplasm (14, 15). MK2 is important in several p38-regulated pathways, because it is a direct substrate for p38 and has been shown to be involved in posttranscriptional regulation of TNF-α in macrophages and rheumatoid synovial cells, IL-6 in macrophages and HeLa cells, and IL-8 in HeLa cells (12, 16–18). Thus, several reports have

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[7] Abbreviations used in this paper: MK2, MAPK-activated protein kinase 2; Hsp27, 27-kDa heat shock protein; KBM, keratinocyte basal medium; RPLP0, ribosomal protein, large, P0; siRNA, small interfering RNA; K-SFM, keratinocyte serum-free medium.

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observed that cells isolated from mice deleted for MK2 are deficient in the LPS-induced biosynthesis of several proinflammatory cytokines regulated by p38, including TNF-α, IL-6, and IL-1 (17, 19, 20). In addition, $MK2^{-/-}$ mice show increased stress resistance and survive LPS-induced endotoxic shock due to a reduction of ~90% in the production of TNF-α (19). In another study, MK2 was demonstrated to play an essential role in host defense against intracellular bacteria via regulation of TNF-α and IFN-γ production (21). MK2 signaling is known to increase TNF-α through translational control via the AU-rich elements of the 3′-untranslated region of TNF-α mRNA (17), whereas in the case of IL-6 and IL-8, MK2 signaling leads to increased mRNA stability (12, 17).

Given that TNF-α is known to play a pivotal role in psoriasis, as demonstrated by the successful treatment of psoriasis by anti-TNF-α Abs (22–24), and the fact that p38 is activated in lesional psoriatic skin, we examined the p38 substrate MK2 in psoriatic skin and its role in cytokine production. We demonstrate for the first time that the increased TNF-α protein expression in lesional psoriatic skin is associated with normal TNF-α mRNA expression. Furthermore, the MK2 activity was found to be significantly augmented in lesional psoriatic skin compared with nonlesional psoriatic skin. In vitro studies of cultured human keratinocytes, using small interfering RNA (siRNA) technology to inhibit MK2 expression, showed that inhibition of MK2 significantly decreased the anisomycin-induced production of the proinflammatory cytokines TNF-α, IL-6, and IL-8, identifying both p38 MAPK and MK2 as potential targets in the treatment of psoriasis and other inflammatory diseases.

Materials and Methods

Biopsies

Keratome biopsies were obtained from lesional and nonlesional psoriatic skin as previously described (25). Briefly, keratome biopsies from lesional and nonlesional plaque-type psoriatic skin were taken from the center of a plaque from patients with moderate to severe chronic stable plaque psoriasis from either the upper or lower extremities. For each patient, biopsies were taken from only one anatomical site. Biopsies were taken as paired samples, and biopsies from nonlesional psoriatic skin were taken from the same body region as biopsies from lesional psoriatic skin at a distance of at least 5 cm from a lesional plaque. Informed consent was obtained from each patient. For Western blotting and kinase assay analysis, the biopsies were taken from the center of a plaque and from nonlesional skin from patients with moderate to severe chronic stable plaque psoriasis.

For immunofluorescence analysis, 4-mm punch biopsies were taken from the center of a chronic plaque and nonlesional psoriatic skin. These biopsies were embedded in paraffin. For quantitative RT-PCR, 4-mm punch biopsies from nonlesional skin, chronic plaques, and acute guttate skin lesions were taken, immediately snap-frozen in liquid nitrogen, and stored in liquid nitrogen until further use. The local ethical committee of Aarhus approved all described studies.

Cell cultures

Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as previously described (26). Second-passage keratinocytes were grown in keratinocyte serum-free medium (K-SFM; Invitrogen Life Technologies). Twenty-four hours before stimulation with either anisomycin or IL-1β, the medium was changed to keratinocyte basal medium (KBM; the same as K-SFM, but without growth factors) in which the cells were stimulated. In some experiments keratinocytes were pretreated with the p38αβ inhibitor SB202190 (10 μM) for 30 min before stimulation. Cells were grown at 37°C in 5% CO_2 in an incubator.

Western blot analysis

Total cell extracts were prepared from keratome biopsies. The biopsies were homogenized in a cell lysis buffer (20 mM Tris-base (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF) and left on ice for 30 min. Then the samples were centrifuged at 10,000 × *g* for 10 min at 4°C, after which the supernatant constituted the cell lysate. Equal protein amounts (as determined by Bradford assay) were separated by SDS-PAGE and blotted onto nitrocellulose membranes (27). Membranes were incubated with phospho-MK2 (recognizing isoforms 1 and 2 of human phospho-MK2; catalogue no. 3041), MK2 (recognizing isoform 1 and 2 of human MK2; catalogue no. 3042), phospho-p38 (catalogue no. 9211), or p38 (catalogue no. 9212) Abs (Cell Signaling Technology) and detected with anti-rabbit IgG-HRP (DakoCytomation) in a standard ECL reaction (Amersham Biosciences) according to the manufacturer's instructions. Densitometric analysis of the band intensity was conducted using Kodak 1D Image analysis software.

In vitro kinase assays

The MK2 kinase activity was performed using anti-MK2 agarose-conjugated beads (Upstate Biotechnology). The agarose beads were washed twice with ice-cold cell lysis buffer (20 mM Trizma-base (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM PMSF) and then immunoprecipitated with 200 μg of protein extract for 1.5 h at 4°C. Immunocomplexes were isolated and washed twice with ice-cold cell lysis buffer and once with ice-cold kinase buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 30 mM $MgCl_2$, 60 μM ATP, and 1 mM DTT). Immunocomplexes were then incubated for 30 min at 30°C with 30 μl of kinase buffer supplemented with 4 μCi of [γ-^{32}P]ATP and 2 μg of 27-kDa heat shock fusion protein (Hsp27). The reaction was stopped by the addition of Laemmli sample buffer and boiled for 5 min. Samples were analyzed by SDS-PAGE, and phosphorylated Hsp27 was detected by autoradiography.

RNA isolation

Punch biopsies were transferred to 1 ml of −80°C cold RNAlater-ICE (Ambion). Samples were kept at −80°C until 24 h before RNA purification, at which time they were transferred to −20°C. Upon RNA purification, biopsies were removed from RNAlater-ICE and transferred to 175 μl of SV RNA lysis buffer supplemented with 2-ME (SV Total RNA Isolation System; Promega) and homogenized. RNA purification, including DNase treatment of the samples, was completed according to the manufacturer's instructions (SV Total RNA Isolation System; Promega). The RNA was stored until further use at −80°C.

Quantitative RT-PCR

For RT we used TaqMan RT reagents (Applied Biosystems). Primers and probes were purchased from Applied Biosystems. TNF-α mRNA expression was analyzed using TaqMan 20X Assays-On-Demand expression assay mix (assay ID: Hs00174128_m1). The probe was a FAM-labeled minor groove binder probe with a nonfluorescent quencher. As housekeeping gene, we used ribosomal protein, large, P0 (RPLP0).

RPLP0 mRNA expression was determined using TaqMan 20X Assays-On-Demand expression assay mix (assay ID: Hs99999902_m1). The probe was a FAM-labeled minor groove binder probe with a nonfluorescent quencher.

PCR Master Mix was TaqMan 2× Universal PCR Master Mix, No AmpErase (Applied Biosystems). Each gene was analyzed in triplicate.

The real-time PCR machine was a Rotorgene-3000 (Corbett Research). Reactions were run once. Relative gene expression levels were determined using the relative standard curve method as outlined in User Bulletin 2 (ABI PRISM 7700 sequencing detection system; Applied Biosystems). Briefly, a standard curve for each gene was made of 4-fold serial dilutions of total RNA from a punch biopsy from a psoriatic plaque. The curve was then used to calculate the relative amounts of target mRNA in the samples.

ELISA

The TNF-α, IL-6, IL-8, and IL-10 levels in the keratinocyte culture supernatants were measured as follows. TNF-α was measured using a TNF-α DuoSet ELISA Development kit (R&D Systems; catalogue no. DY210) according to the manufacturer's protocol. IL-6 and IL-8 were measured using an ELISA kit from BioSource International (catalogue nos. CHC1263 and CHC1304, respectively), according to the manufacturer's protocol. IL-10 was measured by a sandwich ELISA using a combination of an mAb (catalogue no. MAB217; R&D Systems) and a biotinylated polyclonal Ab (catalogue no. BAF217; R&D Systems), both against IL-10. The final result was determined using an ELISA reader (Laboratory Systems; iEMS Reader MF) at 450 nm. All measurements were performed twice.

Small iRNA transfection

MK2 siRNA against MK2-1 (accession no. NM_004759) and MK2-2 (accession no. NM_32960) were designed by Qiagen. The following probes were designed encoding the desired strands: MK2-1, r(CCAUCAUCGAUGACUACAA)dTdT (sense) and r(UUGUAGUCAUCGAUGAUGG)dCdG (antisense); and MK2-2, r(ACGAGCAGAUCAAGAUAAA)dTdT (sense) and r(UUUAUCUUGAUCUGCUCGU)dAdG (antisense). In this study cultured human keratinocytes were transfected with 75 nM MK2-1 and 75 nM MK2-2 siRNA. Nonsilencing control siRNA is an irrelevant siRNA with random nucleotides and no known specificity. Transfections of the keratinocytes were made according to the manufacturer's protocol (Qiagen) using RNAiFect transfection reagent. A fluorescein-labeled nontarget siRNA control was used to monitor transfection efficiency. MK2 siRNA transfection caused no unintentional activation of the IFN response as determined by ELISA.

Immunofluorescence analysis

Four-micrometer sections of paraffin-embedded tissue samples from lesional and nonlesional psoriatic skin were used. The samples were deparaffinized and then heated at 95°C for 10 min in 10 mM sodium citrate buffer (pH 6.0) for Ag unmasking. The samples were then blocked for 1 h in blocking buffer (PBS containing 0.3% Triton X-100, 0.5% skimmed milk powder, and 1% fish gelatin) before being incubated with anti-phospho-MK2 Ab in blocking buffer overnight at 4°C. The samples were washed and incubated with AlexaFluor 594 secondary Ab (Molecular Probes) for 2 h, washed, and incubated with anti-keratin 14 Ab (mAb LL002; from Dr. I. Leigh, Queen Mary, University of London, London, U.K.) directly conjugated to AlexaFluor 488 (Molecular Probes). Nuclear staining was performed by embedding samples in Prolong Gold antifade reagent with DAPI (Molecular Probes). Samples were viewed using an epifluorescence microscope (Leica).

As a negative control, sections were incubated with blocking buffer without primary Ab or with the respective preimmune sera.

Statistical analysis

For statistical analysis, Student's t test was performed. To test for normal distribution, a probability test was made. A value of $p < 0.05$ was regarded as statistically significant.

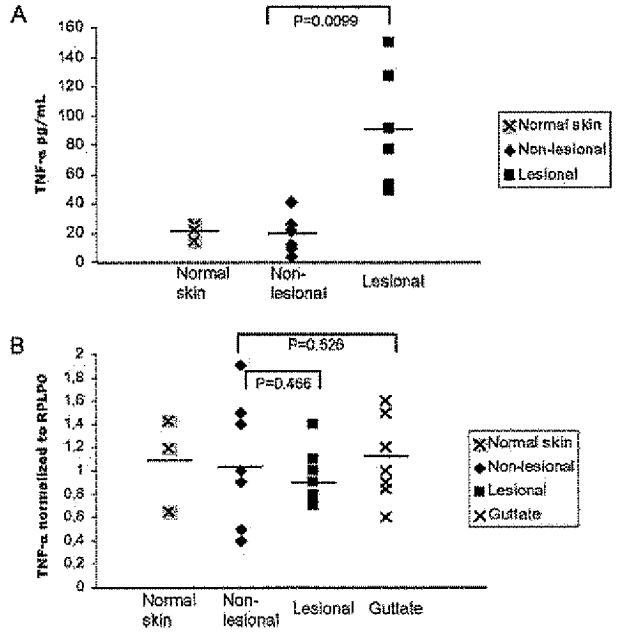

FIGURE 1. TNF-α expression in psoriatic skin. *A*, The TNF-α protein level is significantly increased in lesional psoriatic skin compared with nonlesional psoriatic skin. TNF-α ELISA was performed on cell extracts from keratome biopsies from normal skin (three different subjects) and nonlesional and lesional psoriatic skin (six different psoriatic patients). Each value was determined twice. *B*, The TNF-α mRNA expression was not altered among normal skin, nonlesional, lesional, and guttate psoriatic skin. Punch biopsies from normal skin (three different subjects) and paired punch biopsies from nonlesional, lesional, and guttate psoriatic skin (seven psoriatic patients) were analyzed for TNF-α mRNA by quantitative RT-PCR. The TNF-α mRNA expression was normalized to RPLP0.

Results

TNF-α mRNA and protein expression in psoriatic skin

In accordance with previous studies, we found that the TNF-α protein level was significantly increased in lesional psoriatic skin compared with nonlesional psoriatic skin ($p < 0.01$). In Fig. 1*A*, the results from six psoriatic patients are depicted, showing a mean 4.5-fold increase in the TNF-α protein content in lesional compared with nonlesional psoriatic skin (Fig. 1*A*). The TNF-α protein level in normal skin was comparable to that in nonlesional psoriatic skin. Guttate/acute psoriasis is made up of small lesions, which do not provide enough material to perform Western blot analysis. Therefore, TNF-α protein expression was not determined in these lesions.

Quantitative RT-PCR analysis was performed to investigate whether the increased TNF-α protein level was paralleled by an increased accumulation of the corresponding mRNA in psoriatic skin. Interestingly, no difference in TNF-α mRNA expression was found among normal skin, nonlesional psoriatic skin, chronic plaques, and acute guttate psoriatic skin lesions (Fig. 1*B*), demonstrating a posttranscriptional regulation of TNF-α expression in psoriatic skin.

Immunofluorescence staining of activated MK2 in psoriatic skin

Due to the expression profile of TNF-α in psoriatic skin and the fact that MK2 is known to regulate TNF-α expression at a posttranscriptional level, we examined MK2 activation and localization in lesional and nonlesional psoriatic skin. Single positive cells strongly stained for phospho-MK2 were found in lesional psoriatic skin (Fig. 2, *G* and *H*), but not in nonlesional psoriatic skin (Fig. 2, *C* and *D*). These positively stained cells were scattered throughout the basal and suprabasal layers of the epidermis, and phospho-MK2 was mainly located in the cytoplasm of these cells (Fig. 2, *G–I*). Double staining with keratin 14, a specific keratinocyte marker, showed that phospho-MK2-positive cells also stained positively for keratin 14 (Fig. 2, *H* and *I*).

MK2 is activated in lesional psoriatic skin

TNF-α has been demonstrated to be regulated at a posttranscriptional level by MK2 (19). We therefore analyzed paired keratome biopsies taken from nonlesional and lesional psoriatic skin for MK2 activity. By Western blot analysis using an Ab recognizing the phosphorylated form of MK2, we demonstrated that the phosphorylated form of MK2 was significantly increased in lesional compared with nonlesional psoriatic skin (3.2-fold; $p < 0.05$). No change was seen in the total protein level of MK2 (Fig. 3*A*). The increased level of phosphorylated MK2 in psoriatic skin was paralleled by an increased kinase activity of MK2 in lesional psoriatic skin compared with nonlesional psoriatic skin, as measured by Hsp27 phosphorylation induced by immunoprecipitated MK2 in a kinase assay (2.8-fold; $p < 0.05$; Fig. 3*B*).

Anisomycin and IL-1β activate p38 and MK2, leading to increased TNF-α protein expression

To further analyze the p38/MK2 signaling pathway in epidermis, we used cultured normal human keratinocytes stimulated with anisomycin, a well-characterized p38 activator, or IL-1β. Within 5 min, anisomycin induced rapid activation/phosphorylation of both p38 and MK2, as determined by Western blotting. After 3 h, anisomycin-induced MK2 phosphorylation had almost returned to the

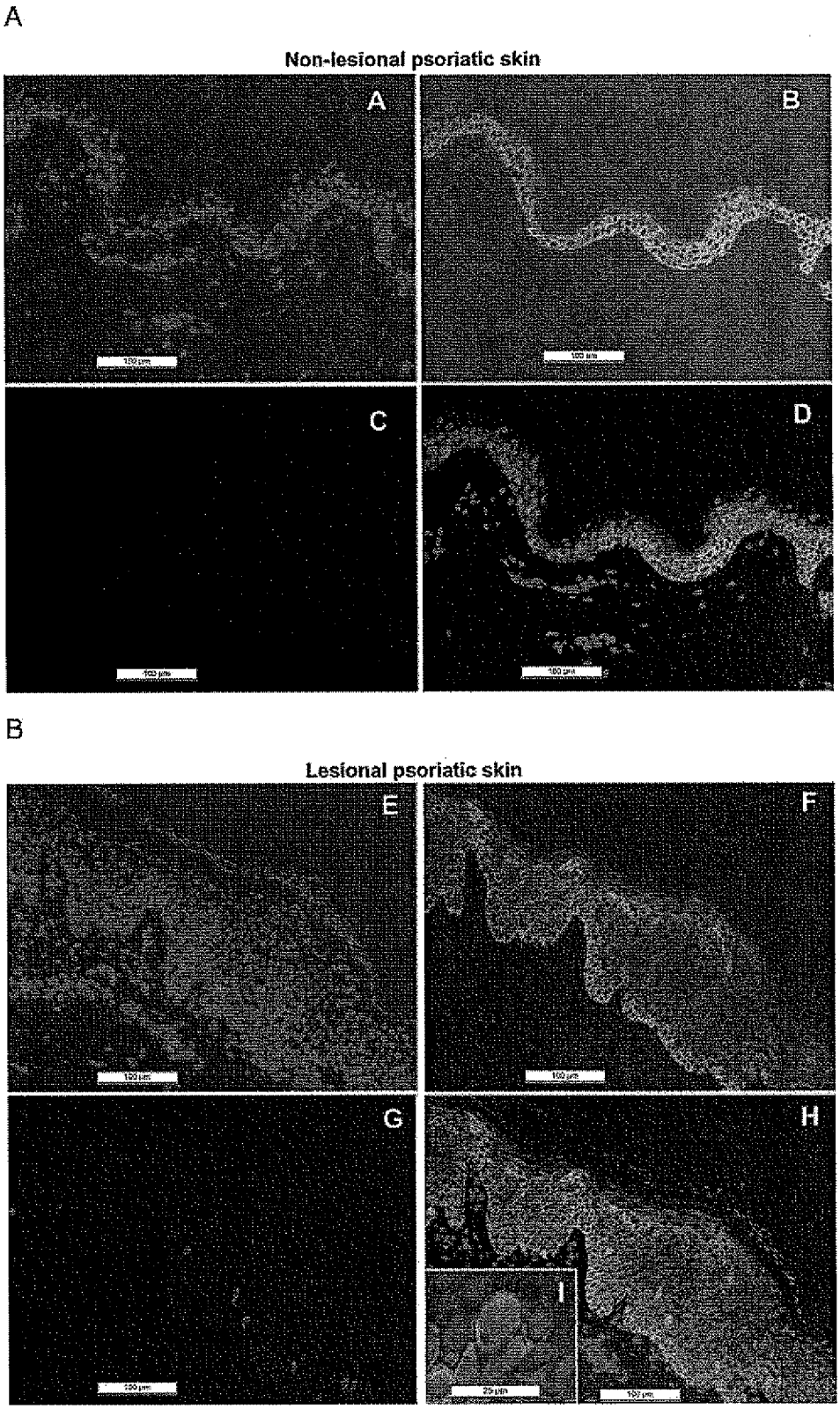

FIGURE 2. Localization of activated MK2 in lesional and nonlesional psoriatic skin. Increased activation of MK2 was found in lesional psoriatic skin, as determined by immunofluorescence staining of nonlesional (*A–D*) and lesional (*E–I*) psoriatic skin using a phospho-MK2-specific primary Ab recognizing phospho-MK2 isoforms 1 and 2. Nuclear staining was performed using 4′,6-diamido-2-phenylindole hydrochloride (blue; *A* and *E*). Green (AlexaFluor 488) demonstrates keratin 14 (*B* and *F*), and red (AlexaFluor 594) demonstrates activated MK2 (*C* and *G*). Yellow indicates colocalization (*H* and *I*). These results were conducted on biopsies from six different patients.

basal level, whereas p38 was still clearly activated even after 3 h (Fig. 4*A*). IL-1β also induced a rapid phosphorylation of both p38 and MK2. The IL-1β-induced phosphorylation of MK2 returned to the basal level after 1 h, whereas the IL-1β-induced phosphorylation of p38 returned to the basal level after 3 h (Fig. 4*A*). Equal protein loading was determined by assessing the total protein amount of p38.

It has been demonstrated that inhibition of the p38/MK2 signaling pathway leads to decreased TNF-α protein production in macrophages (18, 28). To determine whether the anisomycin- and IL-1β-induced phosphorylation of p38 and MK2 led to increased TNF-α protein production in cultured human keratinocytes, TNF-α expression was analyzed by an ELISA. The level of TNF-α was significantly increased ($p < 0.01$) after stimulation with

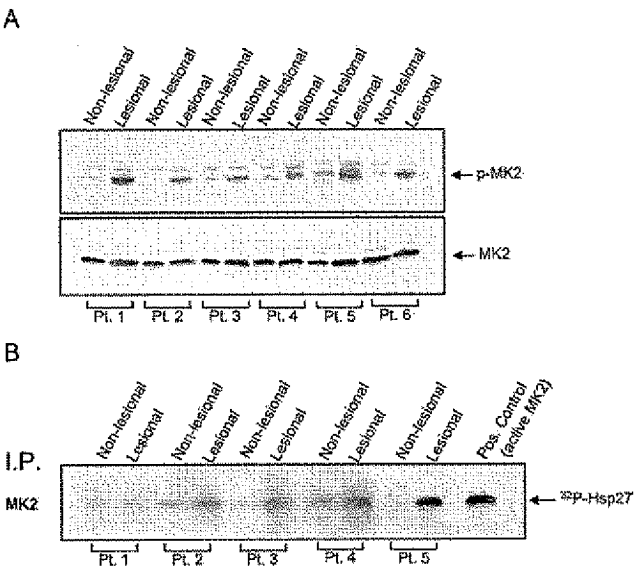

FIGURE 3. MK2 activity in psoriatic skin. *A*, Whole-cell protein extracts were prepared from lesional and nonlesional psoriatic skin and analyzed by Western blotting. The proteins were separated on an 8–16% gradient gel and blotted to a nitrocellulose membrane. After blotting, the membrane was probed with an Ab recognizing the phosphorylated form of both MK2 isoforms 1 and 2. These experiments demonstrated increased levels of the phosphorylated form of MK2 in lesional compared with nonlesional psoriatic skin. *B*, Whole-cell protein extracts (200 μg of protein) from lesional and nonlesional psoriatic skin were prepared for immunoprecipitation of MK2 (isoforms 1 and 2). Enzyme activities were determined by an in vitro kinase assay, using Hsp27 as the substrate. Kinase reactions were prepared for SDS-PAGE, and phosphorylated Hsp27 was detected by autoradiography. Increased kinase activity of MK2 was found in lesional compared with nonlesional psoriatic skin.

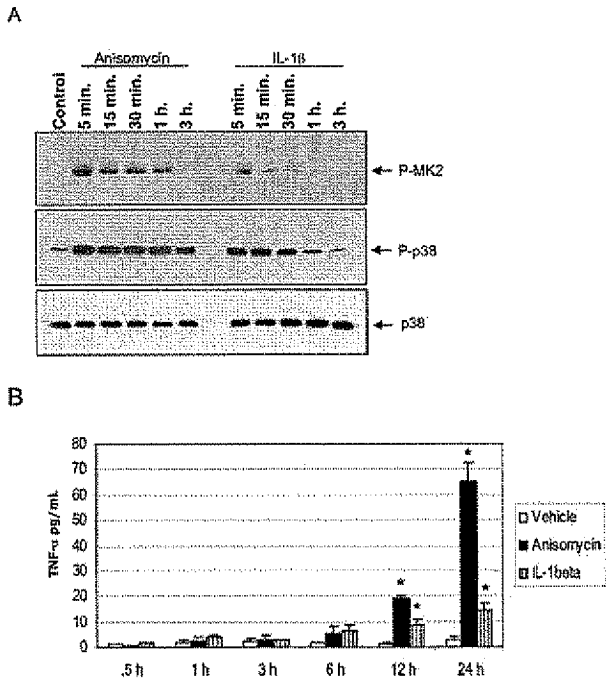

FIGURE 4. Effects of anisomycin and IL-1β on MK2, p38, and TNF-α. *A*, Cultured human keratinocytes were simulated with anisomycin (0.5 μg/ml) or IL-1β (10 ng/ml) for the indicated times. The whole-cell extracts were isolated, and the proteins were separated by SDS-PAGE on an 8–16% gradient gel. After electroblotting, the separated proteins were probed with anti-phospho-MK2 (recognizing the phosphorylated form of MK2 isoforms 1 and 2), anti-phospho-p38, and anti-p38. Both anisomycin and IL-1β led to increased phosphorylation of MK2 and p38 in a time-dependent manner. *B*, Cultured human keratinocytes were stimulated with anisomycin or IL-1β. The supernatant was isolated at the indicated times, and the TNF-α protein content was analyzed by ELISA. Results represent the mean ± SD from four separate experiments. TNF-α protein production in keratinocytes was significantly increased by anisomycin and IL-1β after 12 and 24 h of stimulation. All measurements were performed twice. *, $p <$ 0.01 compared with vehicle-treated cells.

anisomycin or IL-1β for 12 and 24 h. At 24 h, anisomycin-induced TNF-α production had increased ~15-fold, whereas IL-1β-induced TNF-α production had increased ~5-fold compared with vehicle-treated cells (Fig. 4*B*).

Anisomycin- and IL-1β-induced TNF-α production is dependent on p38 MAPK activation

Previous results have demonstrated p38 MAPK to be involved in the regulation of TNF-α in monocytes and macrophages (28–30). To examine whether anisomycin- and IL-1β-induced TNF-α production in cultured human keratinocytes was dependent on p38 MAPK activation, we preincubated keratinocytes with the specific p38α and p38β MAPK inhibitor SB202190 (10 μM) for 30 min before stimulation for 5 min with either anisomycin or IL-1β. Both anisomycin- and IL-1β-induced p38 activations were significantly ($p < 0.05$) reduced by SB202190, as determined by Western blotting (Fig. 5*A*). Measuring TNF-α protein expression by ELISA, we demonstrated that anisomycin-induced TNF-α protein production was significantly inhibited by preincubation of keratinocytes with SB202190 ($p < 0.01$), whereas IL-1β-induced TNF-α protein expression was only moderately, and not significantly, inhibited by SB202190 (Fig. 5*B*).

MK2 is involved in the anisomycin- and IL-1β-induced TNF-α protein production

Having shown the decisive role of p38 in anisomycin-induced TNF-α protein production, we then examined whether MK2 was involved in the induction of TNF-α. This was achieved using siRNA technology to silence MK2 gene expression in cultured human keratinocytes. In keratinocytes transfected with MK2 siRNA target sequences for 48 h, the MK2 protein content was reduced by 85 ± 6% compared with keratinocytes transfected with control siRNA (Fig. 6*A*).

To analyze the role of MK2 in TNF-α protein production, we transfected cultured normal human keratinocytes with MK2 siRNA for 48 h, and then the keratinocytes were stimulated with either anisomycin or IL-1β for another 24 h. Stimulating keratinocytes transfected with control siRNA with anisomycin or IL-1β resulted in significantly increased TNF-α protein production of ~12-fold ($p = 0.00079$) and 4-fold ($p = 0.0047$), respectively, compared with vehicle-treated cells (Fig. 6, *B* and *C*). When keratinocytes were transfected with MK2 siRNA, anisomycin-induced TNF-α protein production was significantly diminished, with an inhibition of ~55% ($p = 0.00031$) compared with keratinocytes transfected with control siRNA (Fig. 6*B*); in contrast to p38 MAPK inhibition, IL-1β-induced TNF-α production was also significantly reduced with an inhibition of ~41% ($p = 0.0017$; Fig. 6*C*).

Anisomycin-induced production of the proinflammatory cytokines IL-6 and IL-8 is mediated through a MK2-dependent mechanism

In addition to TNF-α, IL-6 and IL-8 have been described to be regulated at a posttranscriptional level through the p38/MK2 signaling pathway (12). Therefore, the anisomycin-induced IL-6,

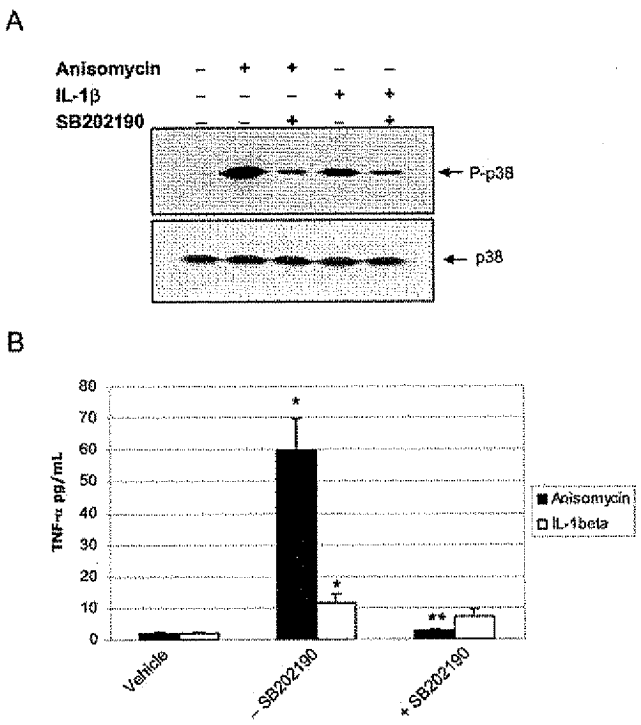

FIGURE 5. Effect of SB202190 on TNF-α protein production. *A*, Anisomycin- and IL-1β-induced phosphorylation of p38 was inhibited by the p38 inhibitor, SB202190. Whole-cell extracts were isolated from keratinocytes preincubated with or without SB202190 (10 μM) for 30 min before being stimulated with anisomycin (0.5 μg/ml) or IL-1β (10 ng/ml) for an additional 5 min. Proteins were separated on an 8–16% gradient gel before being electroblotted onto a nitrocellulose membrane. Active p38 was detected by an anti-phospho-p38 Ab. *B*, The TNF-α protein production induced by anisomycin and IL-1β in keratinocytes was inhibited by SB202190. Cultured keratinocytes were preincubated with SB202190 for 30 min and then stimulated with anisomycin (0.5 μg/ml) or IL-1β (10 ng/ml) for 24 h. The supernatant was isolated, and the TNF-α protein content was determined by ELISA. Results represent the mean ± SD from four separate experiments. All measurements were performed twice. *, $p < 0.01$ compared with vehicle-treated keratinocytes; **, $p < 0.01$ compared with keratinocytes stimulated with anisomycin and without SB202190.

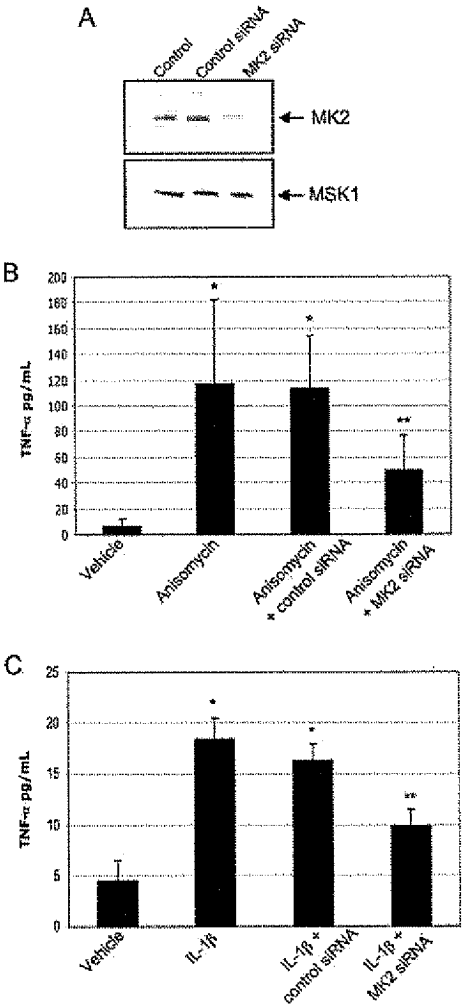

FIGURE 6. MK2-dependent TNF-α protein production. Keratinocytes were transfected with nonspecific control siRNA or specific siRNA for MK2. *A*, Transfection of keratinocytes with MK2 siRNA led to a pronounced inhibition of MK2 protein production. Keratinocytes were cultured for 48 h before whole-cell extracts were isolated. Western blot analysis was performed using an anti-MK2 Ab recognizing both MK2 isoforms 1 and 2. Equal loading was confirmed by incubation with an anti-MSK1 Ab. *B* and *C*, The anisomycin- and IL-1β-induced TNF-α protein production was significantly inhibited by MK2 siRNA. Twenty-four hours after transfection, the medium was changed from K-SFM to KBM, in which keratinocytes were cultured for another 24 h before being stimulated with anisomycin (0.5 μg/ml) or IL-1β (10 ng/ml). Twenty-four hours after stimulation, the supernatant was isolated, and the TNF-α protein content was determined by ELISA. Results represent the mean ± SD from experiments conducted on six different keratinocyte cultures. All measurements were performed twice. *, $p < 0.01$ compared with vehicle-treated keratinocytes; **, $p < 0.01$ compared with keratinocytes transfected with nonspecific control siRNA and stimulated with anisomycin (*B*) or IL-1β (*C*).

IL-8, and IL-10 protein production in human keratinocytes transfected with or without MK2 siRNA was determined. As depicted in Fig. 7, *A* and *B*, protein production of the proinflammatory cytokines IL-6 and IL-8 was significantly diminished, with an inhibition of ~32% ($p = 0.037$) and 50% ($p = 0.0091$), respectively (Fig. 7, *A* and *B*). In contrast, protein expression of the anti-inflammatory cytokine IL-10 was not inhibited by MK2 siRNA, although IL-10 protein expression was significantly induced by anisomycin ($p = 0.0034$; Fig. 7*C*).

Discussion

The proinflammatory cytokine TNF-α is a major mediator of inflammation. TNF-α is not only essential in the pathogenesis of psoriasis. Increased levels of TNF-α have also been found in rheumatoid arthritis and Crohn's disease (31–33). Furthermore, a key role for this cytokine in the pathogenesis of psoriasis, rheumatoid arthritis, and Crohn's disease has been demonstrated by the successful treatment of these diseases with TNF-α antagonists (24, 34, 35). In this study we showed that the protein expression of TNF-α was significantly augmented in lesional psoriatic skin compared with nonlesional psoriatic skin. This is in accordance with previous studies demonstrating increased immunoreactivity and bioactivity of TNF-α in psoriatic skin (36) together with increased TNF-α expression in psoriatic skin, as determined by immunohistochemistry (37). Interestingly, the increased TNF-α protein expression was not paralleled by increased TNF-α mRNA expression in either acute or chronic lesional psoriatic skin compared with nonlesional psoriatic skin. These results demonstrate for the first time that TNF-α is regulated at a posttranscriptional level in psoriatic skin. Previous reports have demonstrated that the p38/MK2 signaling pathway plays a pivotal role in the synthesis of proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8 at a posttranscriptional level (12, 17, 19, 29). We recently demonstrated increased

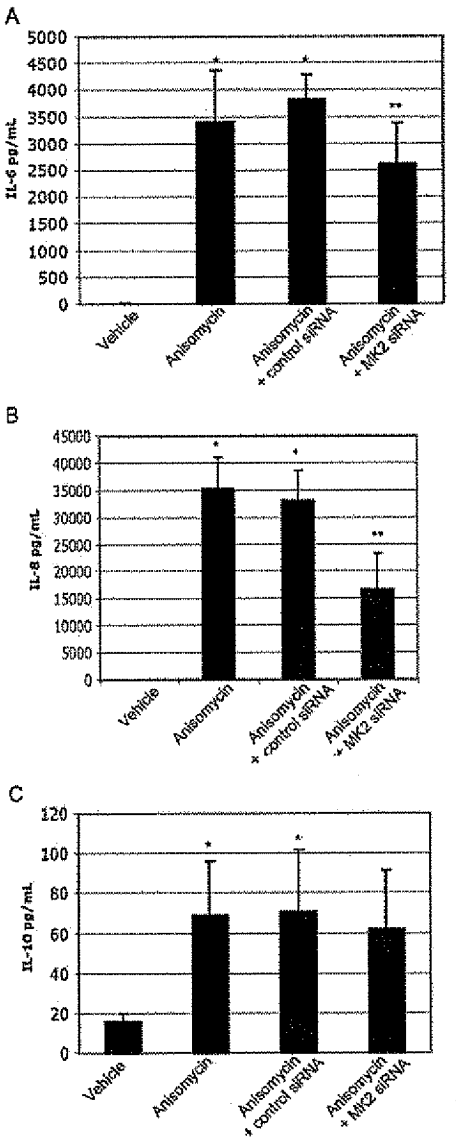

FIGURE 7. Inhibition of IL-6 and IL-8 protein production by MK2 siRNA. Transfection of keratinocytes with MK2 siRNA significantly inhibited the anisomycin-induced IL-6 and IL-8 protein production, but not the IL-10 protein production. Keratinocytes were transfected with nonspecific control siRNA or MK2-specific siRNA. Twenty-four hours after transfection, the medium was changed to KBM, and keratinocytes were cultured for another 24 h before being stimulated with anisomycin (0.5 μg/ml). Twenty-four hours after stimulation, the supernatants were isolated, and the IL-6 (*A*), IL-8 (*B*), and IL-10 (*C*) protein contents were determined by ELISA. Results represent the mean ± SD from experiments conducted on three (*A*), five (*B*), and three (*C*) different keratinocyte cultures, respectively. All measurements were performed twice. *, $p < 0.01$ compared with vehicle-treated keratinocytes; **, $p < 0.05$ compared with keratinocytes transfected with nonspecific control siRNA and stimulated with anisomycin.

kinase activity of the p38 MAPK in lesional compared with nonlesional psoriatic skin (8). Therefore, we examined the activity of MK2, a downstream target for p38 MAPK, and found an increased level of the phosphorylated form of MK2 together with increased kinase activity of MK2 in lesional compared with nonlesional psoriatic skin. In some patients there was a weak positive band for phospho-MK2 in nonlesional psoriatic skin. This may illustrate that the p38 MAPK/MK2 signaling pathway plays a role in maintaining homeostasis in normal skin, whereas it leads to inflammation when it becomes overactivated. Furthermore, detectable basal levels of both TNF-α mRNA and protein were demonstrated in nonlesional psoriatic skin and normal skin (Fig. 1, *A* and *B*). Activated MK2 was selectively expressed in single keratinocytes in the basal and suprabasal layers of lesional psoriatic epidermis, whereas no positive cells were found in nonlesional psoriatic epidermis. Activated MK2 was mainly located in the cytoplasm of the specifically stained cells, in accordance with previous reports demonstrating that MK2 is translocated from the nucleus to the cytoplasm when activated (15).

To further characterize the p38 MAPK/MK2 signaling pathway in epidermal keratinocytes, in vitro studies were conducted with cultured normal human keratinocytes. MK2 has previously been examined in different cell types and cell lines; however, this is the first time that MK2 has been examined in keratinocytes. Both anisomycin and IL-1β caused activation of p38 MAPK and MK2, which led to augmented TNF-α protein expression. The anisomycin-induced TNF-α protein expression was significantly inhibited by the p38 MAPK inhibitor SB202190, demonstrating that anisomycin increases TNF-α by a p38 MAPK-dependent mechanism. In contrast, the IL-1β-induced TNF-α protein expression was only moderately inhibited by SB202190, which could be explained by the fact that anisomycin is known to be a specific p38 MAPK and JNK activator (38), whereas IL-1β, in addition to activation of p38 MAPK and JNK, activates several other kinases, including the MAPKs ERK1 and -2 (39). Because previous reports have demonstrated that MK2 is also a downstream target of ERK1 and -2 in vitro (40, 41), the IL-1β-induced activation of MK2 in keratinocytes may also be mediated by these pathways.

Because specific inhibitors to MK2 are not commercially available, siRNA technology was used to modulate MK2 expression in this study. We demonstrated that specific MK2 siRNA inhibited MK2 expression in cultured human keratinocytes by ~85%. MK2 siRNA significantly inhibited both anisomycin- and IL-1β-induced TNF-α protein expression. The use of siRNA technology led to only partial inhibition of the expression of the specific target protein; this may explain why the anisomycin- and IL-1β-induced TNF-α protein expression was only partially inhibited by ~55 and 41%, respectively. These results demonstrate that MK2 is a more specific target than the p38 MAPK in the inhibition of IL-1β-induced TNF-α protein expression, probably because a number of upstream signaling pathways, including ERK1 and -2 and p38 MAPK, are integrated into the MK2. Interestingly, we also showed that not only TNF-α, but also the anisomycin-induced protein expression of IL-6 and IL-8, was inhibited by MK2 siRNA in cultured human keratinocytes, whereas the anisomycin-induced protein expression of the anti-inflammatory cytokine IL-10 was not inhibited, although IL-10 expression was significantly induced by anisomycin. This strongly indicates that MK2 specifically regulates the translation of proinflammatory cytokines.

This present study is unique because it demonstrates that TNF-α expression is regulated at a posttranscriptional level in psoriasis. Furthermore, we characterize for the first time the localization, expression, and activity of MK2 in lesional and nonlesional psoriatic skin and identify MK2 as the key regulator of TNF-α expression in lesional psoriatic skin as well as in cultured normal human keratinocytes. In vitro data also indicate that MK2 integrate different upstream signaling pathways and induce the expression of predominantly proinflammatory cytokines. These findings are significant because they increase our understanding of how TNF-α expression is regulated in inflammatory conditions such as psoriasis. Based on these findings, we suggest that MK2 may be a new and promising target for specific anti-inflammatory therapy.

Disclosures

The authors have no financial conflict of interest.

References

1. Wrone-Smith, T., and B. J. Nickoloff. 1996. Dermal injection of immunocytes induces psoriasis. *J. Clin. Invest.* 98: 1878–1887.
2. Stern, R. S. 1997. Psoriasis. *Lancet* 350: 349–344.
3. Lew, W., A. M. Bowcock, and J. G. Krueger. 2004. Psoriasis vulgaris: cutaneous lymphoid tissue supports T-cell activation and "type 1" inflammatory gene expression. *Trends Immunol.* 25: 295–305.
4. Bonifati, C., and F. Ameglio. 1999. Cytokines in psoriasis. *Int. J. Dermatol.* 38: 241–251.
5. Zarubin, T., and J. Han. 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell. Res.* 15: 11–18.
6. Kumar, S., J. Boehm, and J. C. Lee. 2003. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat. Rev. Drug Discov.* 2: 717–726.
7. Ono, K., and J. Han. 2000. The p38 signal transduction pathway: activation and function. *Cell. Signal.* 12: 1–13.
8. Johansen, C., K. Kragballe, M. Westergaard, J. Henningsen, K. Kristiansen, and L. Iversen. 2005. The mitogen-activated protein kinases p38 and ERK1/2 are increased in lesional psoriatic skin. *Br. J. Dermatol.* 152: 37–42.
9. Shi, Y., and M. Gaestel. 2002. In the cellular garden of forking paths: how p38 MAPKs signal for downstream assistance. *Biol. Chem.* 383: 1519–1536.
10. Rousseau, S., N. Morrice, M. Peggie, D. G. Campbell, M. Gaestel, and P. Cohen. 2002. Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs. *EMBO J.* 21: 6505–6514.
11. McCormick, C., and D. Ganem. 2005. The kaposin B protein of KSHV activates the p38/MK2 pathway and stabilizes cytokine mRNAs. *Science* 307: 739–741.
12. Winzen, R., M. Kracht, B. Ritter, A. Wilhelm, C. Y. Chen, A. B. Shyu, M. Muller, M. Gaestel, K. Resch, and H. Holtmann. 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J.* 18: 4969–4980.
13. Kyriakis, J. M., and J. Avruch. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* 81: 807–869.
14. Ben-Levy, R., S. Hooper, R. Wilson, H. F. Paterson, and C. J. Marshall. 1998. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr. Biol.* 8: 1049–1057.
15. Engel, K., A. Kotlyarov, and M. Gaestel. 1998. Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation. *EMBO J.* 17: 3363–3371.
16. Campbell, J., C. J. Ciesielski, A. E. Hunt, N. J. Horwood, J. T. Beech, L. A. Hayes, A. Denys, M. Feldmann, F. M. Brennan, and B. M. Foxwell. 2004. A novel mechanism for TNF-α regulation by p38 MAPK: involvement of NF-κB with implications for therapy in rheumatoid arthritis. *J. Immunol.* 173: 6928–6937.
17. Neininger, A., D. Kontoyiannis, A. Kotlyarov, R. Winzen, R. Eckert, H. D. Volk, H. Holtmann, G. Kollias, and M. Gaestel. 2002. MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. *J. Biol. Chem.* 277: 3065–3068.
18. Kotlyarov, A., Y. Yannoni, S. Fritz, K. Laass, J. B. Telliez, D. Pitman, L. L. Lin, and M. Gaestel. 2002. Distinct cellular functions of MK2. *Mol. Cell. Biol.* 22: 4827–4835.
19. Kotlyarov, A., A. Neininger, C. Schubert, R. Eckert, C. Birchmeier, H. D. Volk, and M. Gaestel. 1999. MAPKAP kinase 2 is essential for LPS-induced TNF-α biosynthesis. *Nat. Cell. Biol.* 1: 94–97.
20. Wang, X., L. Xu, H. Wang, P. R. Young, M. Gaestel, and G. Z. Feuerstein. 2002. Mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 deficiency protects brain from ischemic injury in mice. *J. Biol. Chem.* 277: 43968–43972.
21. Lehner, M. D., F. Schwoebel, A. Kotlyarov, M. Leist, M. Gaestel, and T. Hartung. 2002. Mitogen-activated protein kinase-activated protein kinase 2-deficient mice show increased susceptibility to *Listeria monocytogenes* infection. *J. Immunol.* 168: 4667–4673.
22. Gisondi, P., E. Gubinelli, B. Cocuroccia, and G. Girolomoni. 2004. Targeting tumor necrosis factor-α in the therapy of psoriasis. *Curr. Drug Targets Inflamm. Allergy* 3: 175–183.
23. Chew, A. L., A. Bennett, C. H. Smith, J. Barker, and B. Kirkham. 2004. Successful treatment of severe psoriasis and psoriatic arthritis with adalimumab. *Br. J. Dermatol.* 151: 492–496.
24. Gottlieb, A. B., R. Evans, S. Li, L. T. Dooley, C. A. Guzzo, D. Baker, M. Bala, C. W. Marano, and A. Menter. 2004. Infliximab induction therapy for patients with severe plaque-type psoriasis: a randomized, double-blind, placebo-controlled trial. *J. Am. Acad. Dermatol.* 51: 534–542.
25. Johansen, C., E. Flindt, K. Kragballe, J. Henningsen, M. Westergaard, K. Kristiansen, and L. Iversen. 2005. Inverse regulation of the nuclear factor-κB binding to the p53 and interleukin-8 κB response elements in lesional psoriatic skin. *J. Invest. Dermatol.* 124: 1284–1292.
26. Kragballe, K., L. Desjarlais, and C. L. Marcelo. 1985. Increased DNA synthesis of uninvolved psoriatic epidermis is maintained in vitro. *Br. J. Dermatol.* 112: 263–270.
27. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.
28. Kontoyiannis, D., A. Kotlyarov, E. Carballo, L. Alexopoulou, P. J. Blackshear, M. Gaestel, R. Davis, R. Flavell, and G. Kollias. 2001. Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. *EMBO J.* 20: 3760–3770.
29. Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372: 739–746.
30. Young, P., P. McDonnell, D. Dunnington, A. Hand, J. Laydon, and J. Lee. 1993. Pyridinyl imidazoles inhibit IL-1 and TNF production at the protein level. *Agents Actions* 39: C67–C69.
31. Sarzi-Puttini, P., F. Atzeni, Y. Shoenfeld, and G. Ferraccioli. 2005. TNF-α, rheumatoid arthritis, and heart failure: a rheumatological dilemma. *Autoimmun. Rev.* 4: 153–161.
32. Firestein, G. S., J. M. Alvaro-Gracia, and R. Maki. 1990. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J. Immunol.* 144: 3347–3353.
33. Breese, E. J., C. A. Michie, S. W. Nicholls, S. H. Murch, C. B. Williams, P. Domizio, J. A. Walker-Smith, and T. T. MacDonald. 1994. Tumor necrosis factor α-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology* 106: 1455–1466.
34. Maini, R. N., and P. C. Taylor. 2000. Anti-cytokine therapy for rheumatoid arthritis. *Annu. Rev. Med.* 51: 207–229.
35. Rutgeerts, P., G. D'Haens, S. Targan, E. Vasiliauskas, S. B. Hanauer, D. H. Present, L. Mayer, R. A. Van Hogezand, T. Braakman, K. L. DeWoody, et al. 1999. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 117: 761–769.
36. Ettehadi, P., M. W. Greaves, D. Wallach, D. Aderka, and R. D. Camp. 1994. Elevated tumour necrosis factor-α (TNF-α) biological activity in psoriatic skin lesions. *Clin. Exp. Immunol.* 96: 146–151.
37. Nickoloff, B. J., G. D. Karabin, J. N. Barker, C. E. Griffiths, V. Sarma, R. S. Mitra, J. T. Elder, S. L. Kunkel, and V. M. Dixit. 1991. Cellular localization of interleukin-8 and its inducer, tumor necrosis factor-α in psoriasis. *Am. J. Pathol.* 138: 129–140.
38. Hazzalin, C. A., R. Le Panse, E. Cano, and L. C. Mahadevan. 1998. Anisomycin selectively desensitizes signalling components involved in stress kinase activation and fos and jun induction. *Mol. Cell. Biol.* 18: 1844–1854.
39. Saklatvala, J., W. Davis, and F. Guesdon. 1996. Interleukin 1 (IL1) and tumour necrosis factor (TNF) signal transduction. *Philos. Trans. R. Soc. London B* 351: 151–157.
40. Stokoe, D., D. G. Campbell, S. Nakielny, H. Hidaka, S. J. Leevers, C. Marshall, and P. Cohen. 1992. MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.* 11: 3985–3994.
41. Coxon, P. Y., M. J. Rane, S. Uriarte, D. W. Powell, S. Singh, W. Butt, Q. Chen, and K. R. McLeish. 2003. MAPK-activated protein kinase-2 participates in p38 MAPK-dependent and ERK-dependent functions in human neutrophils. *Cell. Signal.* 15: 993–1001.

ORIGINAL ARTICLE

Mitogen- and Stress-Activated Protein Kinase 1 Is Activated in Lesional Psoriatic Epidermis and Regulates the Expression of Pro-Inflammatory Cytokines

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Mitogen- and stress-activated protein kinase 1 (MSK1) is a downstream target of both the p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinases (MAPKs). MSK1 stimulates transcription of different pro-inflammatory genes through activation of transcription factors. The purpose of this study was to investigate the expression and activation of MSK1 in lesional psoriatic skin and its role in cytokine production in cultured normal human keratinocytes. Western blotting revealed a consistent and significant increase in phosphorylated (activated) MSK1(Ser376) in lesional psoriatic skin. Immunofluorescence staining revealed the phosphorylated MSK1(Thr581) to be localized in the basal layers of the epidermis in lesional psoriatic skin. No staining was found in non-lesional psoriatic skin. Cultured human keratinocytes incubated with anisomycin or IL-1β resulted in the phosphorylation of the p38 MAPK and MSK1(Ser376). MSK1(Ser376) phosphorylation was inhibited by pre-incubation with the p38 inhibitor SB 202190. Transfection of the keratinocytes with specific MSK1 small interfering RNA resulted in 80% reduction of MSK1 expression and 51, 40, and 31% decrease in IL-6, IL-8, and tumor necrosis factor-α protein production, respectively. This study demonstrates for the first time the expression of MSK1 in epidermal keratinocytes and increased activation focally in psoriatic epidermis. As MSK1 regulates the production of pro-inflammatory cytokines, it may play a role in the pathogenesis of psoriasis.

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INTRODUCTION

Psoriasis is a chronic immune-mediated inflammatory disease. The prevalence rates are quoted to be around 2% (Christophers, 2001). It is characterized by hyperproliferation and abnormal differentiation of the keratinocytes and infiltration of the skin by activated T cells (Krueger *et al.*, 1990). Abnormalities in several signaling pathways and in the expression or activation of different transcription factors in psoriatic keratinocytes have been hypothesized to play a role in the pathophysiology of psoriasis (McKenzie and Sabin, 2003).

Intracellular protein kinase cascades transduce signals from the cell surface into changes in gene expression. Thereby, they contribute to the regulatory mechanisms controlling cell proliferation, differentiation, and apoptosis. The mitogen-activated protein kinase (MAPK) cascades are among the best characterized of these intracellular protein kinase cascades (Robinson and Cobb, 1997). The p38 MAPK and the extracellular signal-regulated kinase (ERK) 1 and ERK2 MAPKs are two of the best-studied MAPKs.

Mitogen- and stress-activated protein kinase 1 (MSK1) is a downstream effecter kinase of both the p38 and the ERK1/2 MAPKs. MSK1 is predominantly a nuclear enzyme (Deak *et al.*, 1998). It has been shown to phosphorylate various transcription factors including cAMP-response element-binding protein (CREB) (Deak *et al.*, 1998), activation transcription factor 1 (ATF1) (Wiggin *et al.*, 2002), and the p65 subunit of NF-κB at Ser276 (Vermeulen *et al.*, 2003). Lipopolysaccharide-induced transcription of cyclooxygenase 2 and IL-1β is strongly inhibited by the two nonspecific MSK1 inhibitors Ro318220 and H89 in RAW264 macrophages (Caivano and Cohen, 2000), suggesting MSK1 to be involved in the regulation of the expression of these proteins. Another study demonstrated, that IL-6 mRNA levels induced by tumor necrosis factor (TNF) were reduced in the presence of

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Abbreviations: ATF1, activation transcription factor 1; CREB, cAMP-response element-binding protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MSK1, mitogen- and stress-activated protein kinase 1; PBS, phosphate-buffered-saline; real-time qPCR, real-time quantitative PCR; siRNA, small interfering RNA; TNF, tumor necrosis factor

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inhibitors of the p38 and ERK1/2 MAPK pathways as well as in the presence of H89 (Vermeulen *et al.*, 2003).

MSK1/2 double knockout mice are viable and fertile and have no obvious health problems (Wiggin *et al.*, 2002). Embryonic fibroblasts derived from these mice showed a complete abolishment of phosphorylation of CREB and ATF1 in response to anisomycin and UV (Arthur and Cohen, 2000; Wiggin *et al.*, 2002).

We recently reported that the activity of the p38 MAPK and to a lesser degree ERK1/2 MAPKs are increased in lesional psoriatic skin compared with non-lesional skin (Johansen *et al.*, 2005b). The p38 MAPK may, therefore, play an important role in the pathogenesis of psoriasis and other inflammatory conditions. Different inhibitors of the p38 MAPK have been identified and are currently tested in clinical trials of rheumatoid arthritis (Saklatvala, 2004). As the p38 MAPK signaling pathway may serve as a target in the treatment of various inflammatory conditions including psoriasis, it is of interest to further characterize the role of novel p38 MAPK downstream targets in psoriasis.

In this study the localization and activation of MSK1 in lesional psoriatic skin was compared with non-lesional psoriatic skin. We show that the phosphorylated (activated) form of MSK1(Ser376) is significantly increased in lesional psoriatic skin compared with non-lesional skin. We do also, for the first time, use specific MSK1 small interfering RNA (siRNA), to inhibit MSK1 expression, in cultured normal human keratinocytes. This resulted in a significant decrease in the phosphorylation of CREB and in anisomycin-induced expression of the pro-inflammatory cytokines IL-6, IL-8, and TNF-α indicating a role for MSK1 in the production of these cytokines. No alteration was found in anisomycin-induced IFN-γ or IL-1β production. In conclusion, we identify MSK1 as a key kinase in lesional psoriatic skin-regulating IL-6, IL-8, and TNF-α expression in human skin, and therefore, the MSK1 might serve as a putative target in the treatment of psoriasis.

RESULTS

MSK1 expression and activation in lesional psoriatic skin compared with non-lesional psoriatic skin

The phosphorylated Ser376 and total protein expression levels of MSK1 in psoriatic skin were determined by Western blotting. Whole-cell extracts from lesional and non-lesional psoriatic skins were isolated and six psoriatic patients were examined (Figure 1a). Analysis of the band intensity showed a significant increase (3.7-fold, $P<0.05$) in MSK1 Ser376 phosphorylation, and in lesional psoriatic skin compared with non-lesional psoriatic skin (Figure 1a and b). No significant change was found in the band intensity of total MSK1 protein (Figure 1a and c). Equal protein loading was confirmed by assessing the protein level of β-actin (Figure 1a).

Immunofluorescence localization of activated MSK1 in psoriatic skin

We investigated the localization of the Thr581 phosphorylated form of MSK1 by performing immunofluorescence imaging of lesional psoriatic skin and non-lesional psoriatic skin, from six psoriatic patients, obtained as punch biopsies. In all samples studied, a strong staining of Thr581 phospho-MSK1 (activated MSK1) was seen in the lesional psoriatic skin. The staining was present locally in the basal and suprabasal layers of the epidermis (Figure 2g and h). The Thr581 phospho-MSK1-positive cells stained positive to keratin 14 (Figure 2e and h), a specific marker for keratinocytes. The nuclei were localized with 4′,6-diamidine-2′-phenylindole dihydrochloride (Figure 2f and h). The Thr581-phosphorylated MSK1 was mainly localized in the nucleus of these keratinocytes, but some staining was also seen in the cytoplasm (Figure 2h and i). The staining of keratin 14 and the nuclei in non-lesional psoriatic skin is seen in Figure 2a and b, respectively. No staining of phospho-MSK1(Thr581) was detected in the non-lesional psoriatic skin (Figure 2c and d).

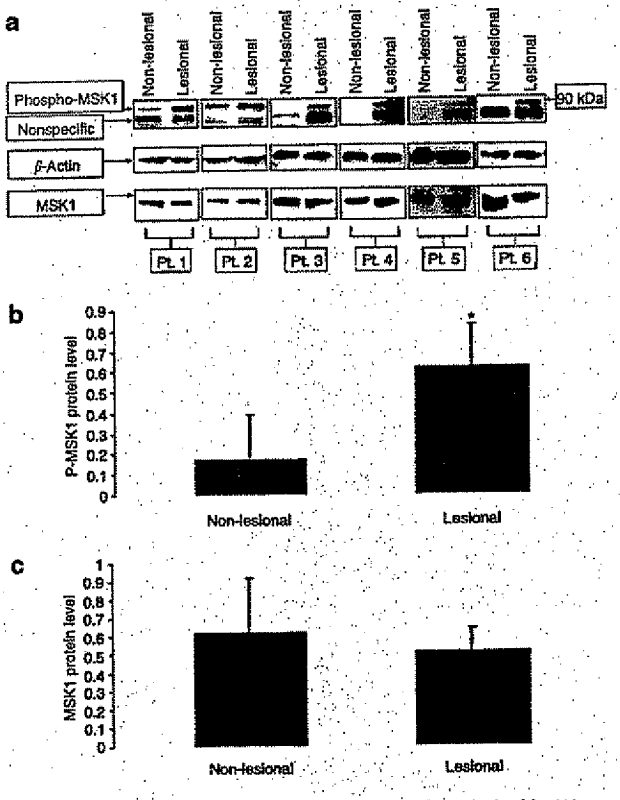

Figure 1. MSK1 activities in lesional and non-lesional psoriatic skin. Western blotting of equivalent amounts of whole-cell protein extracts from non-lesional and lesional psoriatic skin was made. (a) The proteins were loaded onto a 10–20% gradient gel and blotted to a nitrocellulose membrane. The separated proteins were probed with anti-phospho-MSK1(Ser376), or anti-MSK1. Equal loading was confirmed by incubating with anti-β-actin. Densitometric analysis of the band intensity from six patients was carried out and the intensity is presented as mean ± SD (arbitrary units). The level of β-actin was used for normalization. (b) Phospho-MSK1(Ser376) and (c) total MSK1 in non-lesional psoriatic skin compared with lesional psoriatic skin. *$P<0.05$.

IL-1β and anisomycin induces activation of MSK1(Ser376), CREB, and ATF1 in normal human keratinocytes *in vitro*

To study the activation of MSK1(Ser376) in epidermal keratinocytes, cultured normal human keratinocytes were

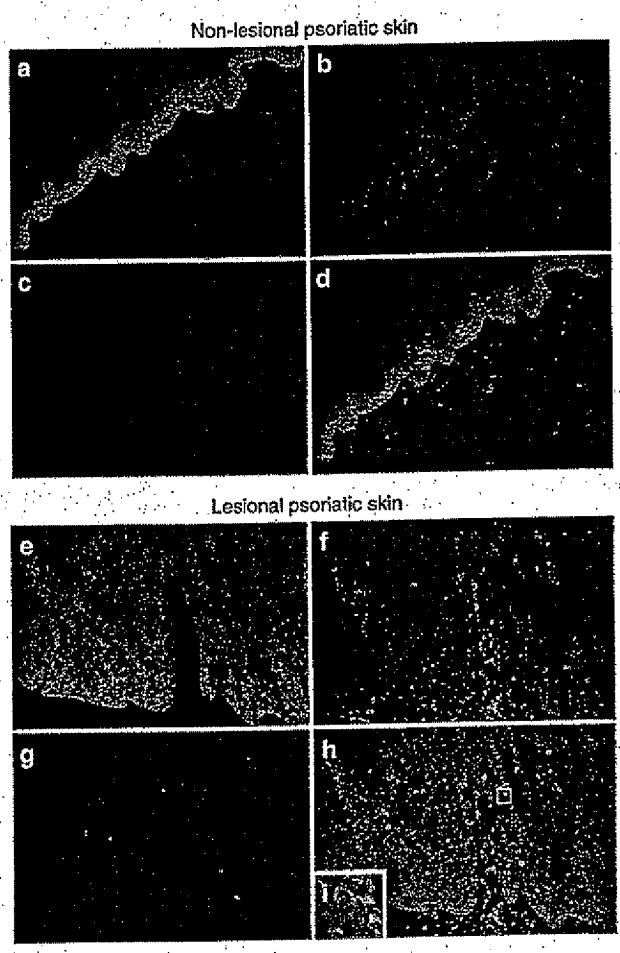

Figure 2. Localization of activated MSK1 in lesional and non-lesional psoriatic skin. Immunofluorescence staining of phospho-MSK1(Thr581) in (**a–d**) non-lesional and (**e–h**) lesional psoriatic skin. Green staining (Alexa Fluor 488) stains keratin-14, blue color (4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI)) stains the nucleus, and red color (Alexa Fluor 594) stains the activated MSK1. The orange color confirms the localization of activated MSK1 in the cytoplasm of specific keratinocytes. Figures show representatives fluorescence images of six psoriatic patients investigated. Original magnification (**a–h**) × 390 and (**i**) × 1,590.

incubated with IL-1β (10 ng/ml) or the specific activator of p38 MAPK anisomycin (0.5 μg/ml), for various times (Figure 3). The levels of phosphorylated-p38 MAPK, -ERK1/2, -MSK1(Ser376), -CREB, and -ATF1 were determined by Western blotting of whole-cell extracts (40 μg of protein). Analysis of the band intensity was carried out for all proteins investigated. A significant increase ($P<0.05$) in the phosphorylated form of ERK1/2, p38, MSK1(Ser376), CREB, and ATF1 was seen after 5 minutes incubation with IL-1β. The level of phosphorylated ERK1/2 and p38 peaked at 5 minutes, whereas the level of phosphorylated MSK1(Ser376), CREB, and ATF1 peaked at 15 minutes (Figure 3).

Stimulation with anisomycin lead to a significant phosphorylation ($P<0.05$) of p38 MAPK, MSK1(Ser376), CREB, and ATF1 seen after 5 minutes, as determined by analysis of

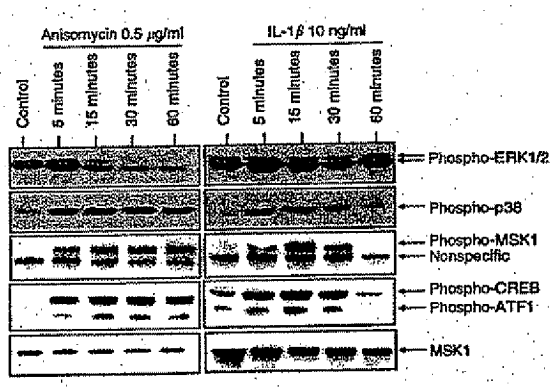

Figure 3. Effects of anisomycin and IL-1β on ERK1/2, p38, MSK1, CREB, and ATF1 phosphorylation. Cultured human keratinocytes were stimulated with IL-1β (10 ng/ml) or anisomycin (0.5 μg/ml) for 5, 15, 30, and 60 minutes. Whole-cell extracts (50 μg of protein) were isolated and separated by SDS-PAGE on a 10–20% gradient gel. After electroblotting, the separated proteins were probed with anti-phospho-ERK1/2 (first serial of bands), anti-phospho-p38 (second serial of bands), anti-phospho-MSK1(Ser376) (third serial of bands), or anti-phospho-CREB/ATF1 (fourth serial of bands). Equal loading was confirmed by incubating with an anti-MSK1 antibody (last band). Representative gels of at least three different experiments are shown.

band intensity, and it was consistent also after 60 minutes (Figure 3). The phosphorylation of these kinases peaked for all of them at 5–30 minutes. The ERK1/2 was not significantly activated by anisomycin.

Equal loading was confirmed by incubation with an anti-MSK1 antibody (Figure 3).

Pre-incubation with SB 202190 significantly inhibits the activation of MSK1(Ser376), CREB, and ATF1 induced by IL-1β and anisomycin

To study the role of p38 in the MSK1 activation, cultured normal human keratinocytes were pre-incubated with SB 202190 (10 μM). The levels of phosphorylated-p38 MAPK, -MSK1(Ser376), -CREB, and -ATF1 were determined by Western blotting of whole-cell extracts (40 μg of protein). Analysis of the band intensity was carried out for all the proteins investigated. Pre-incubation with SB 202190 for 30 minutes before stimulation with either IL-1β (10 ng/ml) or anisomycin (0.5 μg/ml) significantly inhibited ($P<0.05$) the level of phosphorylated-p38 MAPK at 5 minutes of stimulation and -MSK1(Ser376), -CREB and -ATF1 at 15 minutes of stimulation (Figure 4a).

To study the role of ERK1/2 in the anisomycin and IL-1β-induced MSK1 activation, cultured normal human keratinocytes were pre-incubated with PD 98059 (50 μM), a specific inhibitor to ERK1/2 (Pang *et al.*, 1995). The levels of phosphorylated-ERK1/2 MAPKs, -MSK1, -CREB, and -ATF1 were determined by Western blotting of whole-cell extracts (40 μg of protein). Analysis of the band intensity was carried out for all the proteins investigated. Pre-incubation with PD 98059 (50 μM) for 30 minutes before incubation with either IL-1β (10 ng/ml) or anisomycin (0.5 μg/ml) significantly inhibited ($P<0.05$) the level of phosphorylated ERK1/2 at 5 minutes of stimulation. The level of phosphorylated-MSK1(Ser376),

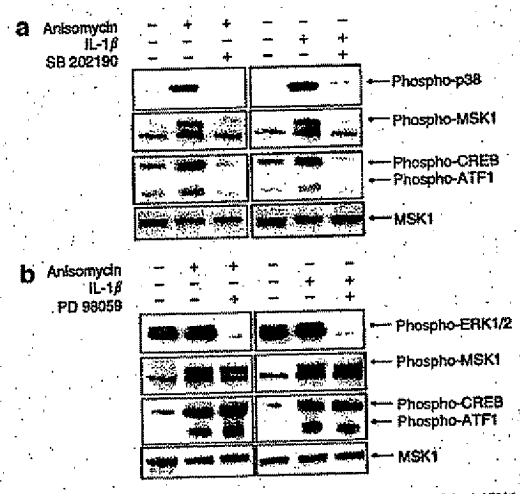

Figure 4. Effects of SB 202190 and PD 98059 on ERK1/2, p38, MSK1, CREB, and ATF1 phosphorylation. (a) Cultured normal human keratinocytes were pretreated with (a) SB 202190 (10 μM) or (b) PD 98059 (50 μM) for 30 minutes before stimulation with IL-1β (10 ng/ml) or anisomycin (0.5 μg/ml) for 5 (phospho-ERK1/2 and phospho-p38 MAPK) and 15 minutes (phospho-MSK1, phospho-CREB, phospho-ATF1). Whole-cell extracts (50 μg of protein) were isolated and separated onto a 10–20% gradient gel. After electroblotting, the separated proteins were probed with (a) anti-phospho-p38, (b) anti-phospho-ERK1/2, (a, b) anti-phospho-MSK1, or (a, b) anti-phospho-CREB/ATF1. Equal loading was confirmed by incubating with an anti-MSK1 antibody. Representative gels of at least three different experiments are shown.

-CREB, and -ATF1 were not significantly inhibited at 15 minutes of stimulation (Figure 4b). However, when the cells were stimulated by IL-1β (Figure 4b), a slight but not significant inhibition of phosphorylated-MSK1(Ser376), -CREB, and -ATF1 was seen. Equal loading was confirmed by incubation with an anti-MSK1 antibody (Figure 4a and b).

MSK1 siRNA transfection reduces MSK1 protein content and the activation of CREB

To study the effect of suppression of the MSK1 gene expression in cultured normal human keratinocytes, the cells were transfected with MSK1 siRNA for 48 hours before stimulation with anisomycin (0.5 μg/ml) for 15 minutes. The levels of total MSK1, MSK2, phosphorylated-CREB, and phosphorylated-ATF2 were determined by Western blotting of whole-cell extracts (40 μg of protein). Analysis of the band intensity was carried out for all the proteins investigated. The mean total MSK1 protein content in the keratinocytes was reduced by approximately 80% ($P<0.05$) in the transfected cells compared to keratinocytes transfected with siControl (Figure 5). The level of phospho-CREB was reduced by approximately 50% ($P<0.05$), whereas no alterations were seen in phospho-ATF2, or total MSK2 protein. The levels of MK2 and β-actin were also examined and no alterations were found (data not shown). The MSK1 siRNA-treated cells were examined for off-target effects (activation of the interferon system) checked by analyzing eIF2α, STAT1, and OAS2 by real-time quantitative PCR (real-time qPCR). No activation of these IFN-associated anti-viral pathway genes was seen (data not shown).

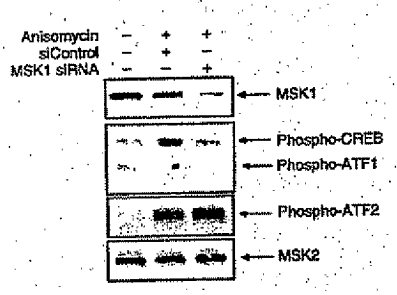

Figure 5. Effects of MSK1 siRNA transfection on total MSK1 protein expression and phosphorylation of CREB. Cultured human keratinocytes were transfected with nonspecific siControl (1 nM) or specific MSK1 siRNA (1 nM) for 5 hours before the medium were changed. The cells were grown for additional 48 hours before stimulation with anisomycin (0.5 μg/ml) for 15 minutes was carried out. Whole-cell extracts (40 μg of protein) were isolated and separated onto a 10–20% gradient gel. After electroblotting, the separated proteins were probed with anti-MSK1, anti-phospho-CREB, or anti-phospho-ATF-2. Equal loading was confirmed by incubating with an anti-MSK2 antibody. Representative gels of at least three different experiments are shown.

The anisomycin-induced IL-6, IL-8, and TNF-α production is regulated through MSK1

To demonstrate the role of anisomycin in IL-6, IL-8, and TNF-α production in human keratinocytes, we stimulated the keratinocytes for 4, 6, and 12 hours. A significant upregulation in expression levels for these cytokines was seen at 4, 6, and 12 hours (Figure 6a–c).

To examine whether MSK1 is involved in the anisomycin-induced IL-6, IL-8, and TNF-α production in human keratinocytes, we analyzed the levels of these cytokines in the MSK1 siRNA-transfected cells. Twelve hours after stimulation, the supernatants were examined by ELISA and Luminex protein array. The total protein content in the cells was used for normalization. We found in all the experiments performed that the cells treated with MSK1 siRNA showed a consistent reduction in IL-6, IL-8, and TNF-α production with the average values of 51% ($P<0.05$), 40% ($P<0.05$), and 31% ($P<0.05$), respectively, compared with the cells treated with nonspecific siRNA (siControl) (Figure 6d–f). The levels of IL-1β and IFN-γ in MSK1 siRNA-treated cells were also investigated and showed no significant changes compared with the cells treated with siControl (data not shown), even though a significant upregulation in these cytokine levels were seen compared with unstimulated cells.

DISCUSSION

MSK1 contribute to the regulation of gene transcription of different pro-inflammatory genes through activation of transcription factors such as CREB, ATF1 and NF-κB (Deak *et al.*, 1998). MSK1 was cloned and characterized in 1998 (Deak *et al.*, 1998; New *et al.*, 1999). Northern blot analysis revealed that MSK1 is ubiquitously expressed, with mRNA found in the eight tissues examined: heart, brain, placenta, lung, kidney, liver, skeletal muscle, and pancreas (New *et al.*, 1999). To our knowledge, MSK1 has never been investigated in the skin, nor has its role in psoriasis been investigated.

In this study, we demonstrate the presence of MSK1 total protein in the lesional psoriatic skin as well as in the

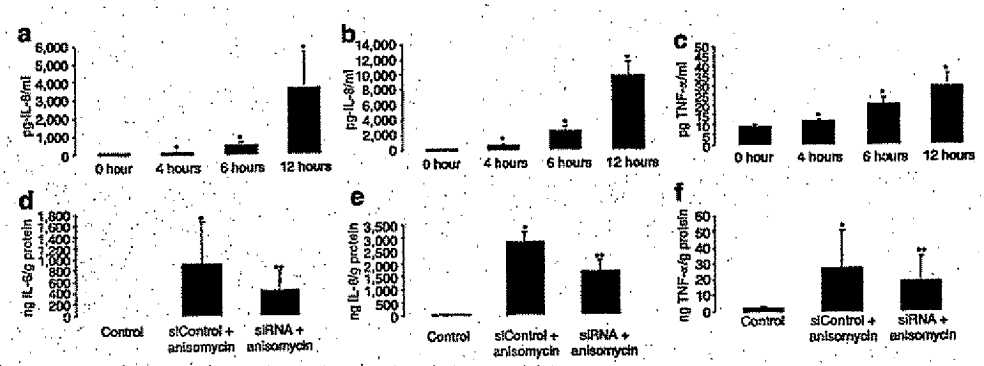

Figure 6. Inhibition of IL-6, IL-8, and TNF-α by MSK1 siRNA. Keratinocytes were stimulated by anisomycin (0.5 μg/ml) for 4, 6, and 12 hours. The (a) IL-6, (b) IL-8, or the (c) TNF-α protein contents were examined by ELISA. Keratinocytes were transfected with siControl (1 nM) or MSK1 siRNA (1 nM) for 48 hours before incubation with anisomycin (0.5 μg/ml). The (d) IL-6, (e) IL-8, or the (f) TNF-α protein contents were examined 12 hours after stimulation by anisomycin (0.5 μg/ml). Results represent mean±SD from at least three different experiments. All measurements were performed in doublets. *$P<0.05$ compared with unstimulated keratinocytes. **$P<0.05$ compared with keratinocytes transfected with siControl.

non-lesional psoriatic skin. We show that the phosphorylated Ser376 and Thr581 forms of MSK1 are increased in lesional psoriatic skin compared with non-lesional psoriatic skin. These two forms has recently been shown to bee essential for the activation of MSK1 (Mccoy *et al.*, 2005) The finding of increased activation of MSK1 in lesional psoriatic skin is interesting, because MSK1 is a downstream target of p38 MAPK (Deak *et al.*, 1998; Roux and Blenis, 2004), and in a very recent publication, we have demonstrated increased p38 MAPK activity in lesional psoriatic skin compared with non-lesional psoriatic skin (Johansen *et al.*, 2005b). Furthermore, MSK1 has been shown to phosphorylate the NF-κB p65 subunit, leading to the stimulation of NF-κB-driven genes (Vermeulen *et al.*, 2003). NF-κB is one of the key regulators of transcription of a variety of genes involved in immune and inflammatory responses. Recently, we demonstrated that the NF-κB DNA-binding activity is regulated in a specific manner in psoriatic skin and that topical treatment of psoriatic skin normalizes this abnormal NF-κB-binding activity (Johansen *et al.*, 2005a). In another recent published study, an upregulation of active phosphorylated NF-κB/rel in the epidermis from psoriatic plaques was demonstrated and a similar downregulation during eternacept treatment (Lizzul *et al.*, 2005). Based on these findings, it is possible that activation of the p38–MSK1–NF-κB signaling pathway plays a role in the pathogenesis of psoriasis.

To further characterize the p38 MAPK/MSK1 signaling pathway in epidermal keratinocytes, *in vitro* studies with cultured normal human keratinocytes were carried out. We showed that keratinocytes express MSK1. Upon stimulation with anisomycin and IL-1β, MSK1(Ser376) as well as CREB and ATF1 activation was detectable already after 5 minutes. These findings are in accordance with results from other cell types (Wiggin *et al.*, 2002; Lee *et al.*, 2003). Our data demonstrate that the levels of the phosphorylated form of MSK1 in the unstimulated control cells are relatively low. A low or absent level of phosphorylated MSK1 has been demonstrated earlier in other cell types. In cortical neurons, phosphorylated MSK1(Ser376) was low, although an activation of ERK also was seen (Arthur *et al.*, 2004). A low level of MSK1 activity in unstimulated cells has also been demonstrated in human umbilical vein endothelial cells (Gustin *et al.*, 2004).

The inhibitor studies demonstrated that the activation of MSK1, CREB, and ATF1 primarily restricts to the p38 MAPK pathway. This is owing to our choice of stimulation agents. Anisomycin and IL-1β are predominantly activating the p38 MAPK pathway of MSK1 rather than the ERK MAPK pathway. We use these activators because of the increased activation of the p38 MAPK in lesional psoriatic skin (Johansen *et al.*, 2005b).

The absence of a specific inhibitor has made it difficult to study the role of MSK1. Previously, H89 and Ro318220 have been used. These two compounds inhibit, however, other kinases, such as protein kinase A/C and casein kinase I (Ravichandran and Burakoff, 1994; Zhong *et al.*, 2001; London, 2003). In the present study, siRNA technology was used for the first time, to specific inhibit MSK1. In cultured keratinocytes stimulated with anisomycin, siRNA inhibited the MSK1 protein content approximately 80% and the phosphorylation of CREB approximately 50%. This is in accordance with the results obtained in anisomycin-stimulated MSK1 "knockout" mice fibroblasts (Arthur and Cohen, 2000; Wiggin *et al.*, 2002). No changes of the closely related proteins MSK2 and MK2, both p38 MAPK downstream targets were detected. In addition, the phosphorylation of the MSK1-independent transcription factor ATF2 was not altered during the transfection.

The inhibition of MSK1 and CREB phosphorylation was accompanied by reduced levels of the anisomycin-induced IL-6, IL-8, and TNF-α, but not of IL-1β and IFN-γ production.

Anisomycin in the concentration used in this study has previously been described to block protein synthesis (Barros *et al.*, 1997). However, we clearly demonstrate that anisomycin in keratinocytes induces cytokine production, which is in accordance with previous results from our group (Johansen *et al.*, 2006).

Several studies indicate the connection between the activation of CREB and IL-6, IL-8, and TNF-α. CREB-binding

sites are found in the promoter regions of TNF-α (Kuprash *et al.*, 1999; Chong *et al.*, 2003). IL-6 and IL-8 production was also found to be dependent on CREB phosphorylation (Ikewaki and Inoko, 2002; Persson *et al.*, 2005). Our findings demonstrate that the expression of selective pro-inflammatory cytokines is partially controlled by MSK1 activity.

The use of siRNA technology only leads to a partial inhibition, a "knockdown" and not a "knockout" of the expression of the specific target protein. This could explain the fact that only a partial inhibition of IL-6, IL-8, and TNF-α expression was achieved during the MSK1 siRNA transfection. However, the incomplete inhibition of IL-6, IL-8, and TNF-α, may also suggest that other kinases, downstream of the p38 MAPK, are involved in the regulation of these cytokines. In particular, the phosphorylation of CREB is known to be dependent on both the MSK1 and MSK2 (Wiggin *et al.*, 2002). Owing to this, it would be of interest to determine whether simultaneous inhibition of the MSK2 might result in a further inhibition of phosphorylated-CREB and these cytokines.

In this study, we demonstrate for the first time the presence of MSK1 in human skin. We demonstrate that MSK1 is activated in lesional psoriatic skin compared with non-lesional psoriatic skin. *In vitro* studies with cultured normal human keratinocytes determine MSK1 to play a pivotal role in phosphorylation of CREB and in IL-6, IL-8, and TNF-α production. Taken together, our data indicate that MSK1 may play a role in the pathophysiology of psoriasis and that the p38/MSK1 signaling pathway may be a novel target in the treatment of psoriasis.

MATERIALS AND METHODS

Biopsies

Keratome biopsies were obtained as described previously (Johansen *et al.*, 2004, 2005b) from lesional psoriatic skin and from non-lesional psoriatic skin. The patients had untreated plaque-type psoriasis and the biopsies were taken from the centre of a plaque with moderate-to-severe psoriasis from either the upper or lower extremities. Furthermore, paired 4 mm punch biopsies were taken from lesional and non-lesional psoriatic skin for immunofluorescence. These biopsies were paraffin-embedded and 4-μm sections were placed on slides.

The study was conducted according to the Declaration of Helsinki Principles. The medical ethical committee of Aarhus approved the study. Informed consent was obtained from each patient.

Cell cultures

Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as described previously (Kragballe *et al.*, 1985; Johansen *et al.*, 2003). Second-passage keratinocytes were grown in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA). The medium was changed to keratinocyte basal medium (the same as keratinocyte serum-free medium, but without growth factors) 24 hours before stimulation. The cells were stimulated with anisomycin (0.5 μg/ml; Sigma Aldrich, St Louis, MO) or IL-1β (10 ng/ml; R&D Systems, Oxon, UK). In some experiments, the keratinocytes were pretreated with the p38α/β inhibitor SB 202190 (10 μM; Calbiochem, San Diego, CA) or the MAPK inhibitor PD 98059 (50 μM; Calbiochem, San Diego, CA) 30 minutes before stimulation. A recent publication demonstrated that SB 202190 is not necessarily specific for p38, but also capable of binding to cholecystokinin 1 (Morel *et al.*, 2005).

Cells were grown at 37°C and 5% CO_2.

Western blot analysis – cells

A solution containing 50 mM Tris-HCl, pH 6.8, 10 mM dithiothretiol, 10 mM β-glycerophosphate, 10 mM sodium flouroide, 0.1 mM sodium orthovanadate, 10% glycerol, 2.5% SDS (BDH), and phenylmethylsulfonyl fluoride and Complete-proteinase inhibitor cocktail were used to the lysation of the cells. The lysates were boiled for 3 minutes, incubated with benzon nuclease for 15 minutes, and then centrifuged at 13,600 *g* for 3 minutes at 4°C and the supernatants were removed and stored at −80°C until use.

Equal amount of whole-cell protein extracts (determined by Bradford) were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were incubated with either anti-phospho-p38, -phospho-ERK, -phospho-MSK1(Ser376), -phospho-CREB, phospho-ATF2, -CREB (Cell Signalling Technology, Beverly, MA), -MSK2 (R&D Systems, Oxon, UK) or anti-MSK1 (Santa Cruz Biotechnology, Santa Cruz, CA), and detected with horseradish peroxidase-conjugated anti-rabbit or anti-goat (DAKO, Glostrup, Denmark) in a standard ECL reaction (Cell Signalling Technology, Beverly, MA). A biotinylated protein ladder molecular weight marker (Cell Signalling Technology, Beverly, MA) was used for the estimation of protein size. Densitometric analysis of the band intensity was carried out using Kodak 1D Image Analysis Software.

Western blot analysis – keratome biopsies

The biopsies were homogenized in a cell lysis buffer (20 mM Tris-Base, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and left on ice for 30 minutes. The samples were centrifuged at 10,000 *g* for 10 minutes at 4°C. The supernatant constitute the cell lysate. The Western blotting was carried out as described in "Western blot analysis – cells".

Immunofluorescence

The sections were deparaffinized in xylene, hydrated through a descending ethanol series, and for antigen unmasking the sections were heated in 10 mM sodium citrate buffer (pH 6.0) for 1 minute at full power and 9 minutes at medium power followed by cooling for 20 minutes. Nonspecific binding sites were blocked by 30 minutes of incubation in blocking phosphate-buffered-saline (PBS) (0.5% non-fat dry milk, 1% fish gelatine, 0.3% Triton X-100). Tissue sections were immunostained with the anti-phospho-MSK1(Thr581) (Cell Signalling Technology, Beverly, MA; no. 9595) overnight at 4°C. The next day, the sections were incubated with the secondary antibody (Alexa Flour 594; Molecular Probes, Eugene, OR) diluted in blocking PBS buffer for 2 hours. The sections were blocked with mouse serum before incubation with the K14 antibody LL002 (a generous gift from Dr I. Leigh, Cancer Research, UK), directly conjugated to Alexa Flour 488 (Molecular Probes, Eugene, OR). Nuclear staining was performed by mounting samples in Prolong Gold anti-fade reagent with 4′,6-diamidine-2′-phenylindole dihydrochloride (Molecular

Probes, Eugene, OR). To ensure that the staining was not owing to nonspecific binding of the secondary antibody or owing to nonspecific binding of rabbit IgG, negative controls were made incubating the slides with either blocking buffer without primary antibody or normal rabbit IgG.

siRNA transfection

Primary keratinocytes were seeded the day before transfection. Cell density was 60–70%. Before transfection, the cells were changed to keratinocyte basal medium. MSK1 siRNA, Pool-number M-004665-01 (Dharmacon, Lafayette, CO), and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were pre-incubated with keratinocyte basal medium. The medium-diluted siRNA and the medium-diluted Lipofectamine 2000 incubated for 20 minutes allowing complex formation. The siRNA:Lipofectamine complexes were added to the cells (final MSK1 siRNA concentration: 1 nM). As control, siControl Non-Targeting siRNA Pool (1 nM) (Dharmacon, Lafayette, CO) was used. Five hours after transfection, the medium was changed to keratinocyte serum-free medium. The cells were grown at 37°C and 5% CO_2.

RNA extraction and real-time qPCR

Total RNA was prepared from human primary keratinocytes by lysing the cells with TRIzol (Invitrogen, Carlsbad, CA). To avoid secondary inhibitory mRNA structures, 1 μg of total RNA was mixed with 50 pmol oligo-dT_{16} (in a total volume of 13 μl) and annealed at a temperature gradient initiating at 80°C and terminating at 50°C. Subsequently, cDNA synthesis was performed using Superscript II according to the supplier's manual (Invitrogen, Carlsbad, CA). The annealed mRNA:oligo-d(T) was reverse transcribed in a solution containing 1 × Superscript II PCR buffer, 20 U RNasin (Promega, Madison, WI), 0.3 mM $MgCl_2$, 0.5 mM dNTP, and 200 U Superscript II in a total volume of 20 μl at 50°C for 60 minutes. The cDNA was diluted with 280 μl water before real-time qPCR measurements. Real-time qPCR using an ABI 7900HT sequence detection system and Taqman PCR primer/probes (Applied Biosystems, Foster City, CA) was performed according to the manufacturer's protocol. Five microliter of the first-strand cDNA was used in an assay consisting of 10 μl Taqman Universal PCR master mix (Applied Biosystems, Foster City, CA), 4 μl water, and 1 μl primer/probe. The following primer/probes from Applied Biosystems (Assay-on-demand) were used to detect the STAT 1 gene expression (Hs01013998_m1), the OAS2 gene expression (Hs00942650_m1), the eIF2α gene expression (Hs01026895_m1), and the glyceraldehyde-3-phosphate dehydrogenase gene expression (Hs99999905_m1). The conditions for the real-time qPCR were as follows: 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All genes are normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

ELISA

IL-8 and TNF-α expression was measured by ELISA using 96 wells Maxisorb (Invitrogen, Carlsbad, CA) plates, and the DuoSet® ELISA Development System (R&D Systems, Oxon, UK) according to manufacturer's instructions (R&D Systems). The wells were incubated with 100 μl diluted Capture Antibody, 4.0 μg/ml, overnight at room temperature. The next day, the wells were washed three times with (PBS) containing 0.05% Tween®20 (PBST), before they were blocked for 1 hour in 300 μl of 1% bovine serum albumin (BSA) in PBST. The standards and the samples were diluted in either 1% BSA in PBS, for the TNF-α ELISA, or in reagent diluent containing 0.1% BSA, 0.05% Tween®20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl, pH 7.2), for the IL-8 ELISA. Hundred microliter were added to the wells and incubated for 2 hours at room temperature. The wells were washed three times with PBS and then incubated at room temperature for 2 hours with 100 μl diluted detection antibody, either 20 ng/ml (IL-8 ELISA) or 75 ng/ml (TNF-α ELISA). The wells were washed three times in PBST, and then they were incubated with 100 μl diluted streptavidin-horseradish peroxidase (1:200) for 20 minutes. The wells were washed again in PBST and visualized by adding 100 μl of substrate solution (R&D Systems, Oxon, UK) and incubated for 20 minutes. The reactions were stopped by incubation with 2 N H_2SO_4. Finally, the results were determined, in doublets, by an ELISA reader (Laboratory Systems iEMS Reader MF, Copenhagen, Denmark) at 450 nm.

Luminex protein array

Expression of IL-6, IL-1β, and IFN-γ was measured using the Luminex Technology™, and a 17-plex human cytokine kit purchased from Bio-Rad. Briefly, the filter plate was washed with assay buffer and 50 μl of freshly vortexed antibody-conjugated beads were added to each well. The plate was washed with assay buffer and samples and standards were added. After a brief shake (30 seconds at 1,100 r.p.m.), the plate was incubated at room temperature in the dark for 45 minutes with light shaking (300 r.p.m.). After one wash-step, 25 μl of the detection antibody was added to each well, and the plate was shaken and incubated as above. Subsequently, the plate was washed and incubated for 10 minutes with 50 μl of a streptavidin-PE solution with shaking (30 seconds at 1,100 r.p.m., 10 minutes at 300 r.p.m.). Finally, the plate was washed and 125 μl of assay buffer was added to each well and the plate shaken for 10 hours at 1,100 r.p.m. and read immediately on the Bio-Plex machine.

Statistics

Results were expressed as mean±standard deviation. Statistical significance ($P<0.05$) was assessed by Student's *t*-test. To test for normal distribution, a probability test was conducted.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Arthur JSC, Cohen P (2000) MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells. *FEBS Lett* 482: 44–8

Arthur JSC, Fong AL, Dwyer JM, Davare M, Reese E, Obrietan K *et al.* (2004) Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. *J Neurosci* 24:4324–32

Barros LF, Young M, Saklatvala J, Baldwin SA (1997) Evidence of two mechanisms for the activation of the glucose transporter GLUT1 by

anisomycin: p38(MAP kinase) activation and protein synthesis inhibition in mammalian cells. *J Physiol (London)* 504:517–25

Caivano M, Cohen P (2000) Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 beta in RAW264 macrophages. *J Immunol* 164:3018–25

Chong YH, Shin YJ, Suh YH (2003) Cyclic AMP inhibition of tumor necrosis factor alpha production induced by amyloidogenic C-terminal peptide of Alzheimer's amyloid precursor protein in macrophages: Involvement of multiple intracellular pathways and cyclic AMP response element binding protein. *Mol Pharmacol* 63:690–8

Christophers E (2001) Psoriasis – epidemiology and clinical spectrum. *Clin Exp Dermatol* 26:314–20

Deak M, Clifton AD, Lucocq JM, Alessi DR (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J* 17:4426–41

Gustin JA, Pincheira R, Mayo LD, Ozes ON, Kessler KM, Baerwald MR *et al.* (2004) Tumor necrosis factor activates CRE-binding protein through a p38 MAPK/MSK1 signaling pathway in endothelial cells. *Am J Physiol Cell Physiol* 286:C547–55

Ikewaki N, Inoko H (2002) A very late activating antigen-alpha 4 (CD49d) monoclonal antibody, BU49 induces phosphorylation of a cAMP response element-binding protein (CREB), resulting in induction of homotypic cell aggregation and enhancement of interleukin-8 (IL-8) production. *Microbiol Immunol* 46:685–95

Johansen C, Flindt E, Kragballe K, Henningsen J, Westergaard M, Kristiansen K *et al.* (2005a) Inverse regulation of the nuclear factor-kappa B binding to the p53 and interleukin-8 kappa B response elements in lesional psoriatic skin. *J Invest Dermatol* 124:1284–92

Johansen C, Funding AT, Otkjaer K, Kragballe K, Jensen UB, Madsen MW *et al.* (2006) Protein expression of TNF-α in psoriatic skin is regulated at a post-transcriptional level by MK2. *J Immunol* 176:1431–8

Johansen C, Kragballe K, Henningsen J, Westergaard M, Kristiansen K, Iversen L (2003) 1 Alpha,25-dihydroxyvitamin D-3 stimulates activator protein 1 DNA-binding activity by a phosphatidylinositol 3-kinase/Ras/MEK/extracellular signal regulated kinase 1/2 and c-Jun N-terminal kinase 1-dependent increase in c-Fos, Fra1, and c-Jun expression in human keratinocytes. *J Invest Dermatol* 120:561–70

Johansen C, Kragballe K, Rasmussen M, Dam TN, Iversen L (2004) Activator protein 1 DNA binding activity is decreased in lesional psoriatic skin compared with nonlesional psoriatic skin. *Br J Dermatol* 151:600–7

Johansen C, Kragballe K, Westergaard M, Henningsen J, Kristiansen K, Iversen L (2005b) The mitogen-activated protein kinases p38 and ERK1/2 are increased in lesional psoriatic skin. *Br J Dermatol* 152:37–42

Kragballe K, Desjarlais L, Marcelo CL (1985) Increased DNA-synthesis of uninvolved psoriatic epidermis is maintained *in vitro*. *Br J Dermatol* 112:263–70

Krueger JG, Krane JF, Carter DM, Gottlieb AB (1990) Role of growth-factors, cytokines, and their receptors in the pathogenesis of psoriasis. *J Invest Dermatol* 94:S135–40

Kuprash DV, Udalova IA, Turetskaya RL, Kwiatkowski D, Rice NR, Nedospasov SA (1999) Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J Immunol* 162:4045–52

Lee CW, Nam JS, Park YK, Choi HK, Lee JH, Kim NH *et al.* (2003) Lysophosphatidic acid stimulates CREB through mitogen- and stress-activated protein kinase-1. *Biochem Biophys Res Commun* 305: 455–61

Lizzul PF, Aphale A, Malaviya R, Sun Y, Masud S, Dombrovskiy V *et al.* (2005) Differential expression of phosphorylated NF-kappa B/RelA in normal and psoriatic epidermis and downregulation of NF-kappa B in response to treatment with etanercept. *J Invest Dermatol* 124: 1275–83

London FS (2003) The protein kinase C inhibitor RO318220 potentiates thrombin-stimulated platelet-supported prothrombinase activity. *Blood* 102:2472–81

Mccoy CE, Campbell DG, Deak M, Bloomberg GB, Arthur JSC (2005) MSK1 activity is controlled by multiple phosphorylation sites. *Biochem J* 387:507–17

McKenzie RC, Sabin E (2003) Aberrant signalling and transcription factor activation as an explanation for the defective growth control and differentiation of keratinocytes in psoriasis: a hypothesis. *Exp Dermatol* 12:337–45

Morel C, Ibarz G, Oiry C, Carnazzi E, Berge G, Gagne D *et al.* (2005) Cross-interactions of two p38 mitogen-activated protein (MAP) kinase inhibitors and two cholecystokinin (CCK) receptor antagonists with the CCK1 receptor and p38 MAP kinase. *J Biol Chem* 280:21384–93

New L, Zhao M, Li YQ, Bassett WW, Feng Y, Ludwig S *et al.* (1999) Cloning and characterization of RLPK, a novel RSK-related protein kinase. *J Biol Chem* 274:1026–32

Pang L, Sawada T, Decker SJ, Saltiel AR (1995) Inhibition of Map kinase kinase blocks the differentiation of Pc-12 cells induced by nerve growth-factor. *J Biol Chem* 270:13585–8

Persson E, Voznesensky OS, Huang YF, Lerner UH (2005) Increased expression of interleukin-6 by vasoactive intestinal peptide is associated with regulation of CREB, AP-1 and C/EBP, but not NF-kappa B, in mouse calvarial osteoblasts. *Bone* 37:513–29

Ravichandran KS, Burakoff SJ (1994) Evidence for differential intracellular signaling via Cd4 and Cd8 molecules. *J Exp Med* 179:727–32

Robinson MJ, Cobb MH (1997) Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 9:180–6

Roux PP, Blenis J (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 68:320–44

Saklatvala J (2004) The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Curr Opin Pharmacol* 4:372–7

Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G (2003) Transcriptional activation of the NF-kappa B p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO J* 22:1313–24

Wiggin GR, Soloaga A, Foster JM, Murray-Tait V, Cohen P, Arthur JSC (2002) MSK1 and MSK2 are required for the mitogen-and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. *Mol Cell Biol* 22:2871–81

Zhong SP, Jansen C, She QB, Goto H, Inagaki M, Bode AM *et al.* (2001) Ultraviolet B-induced phosphorylation of histone H3 at serine 28 is mediated by MSK1. *J Biol Chem* 276:33213–9

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The mitogen-activated protein kinases p38 and ERK1/2 are increased in lesional psoriatic skin

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Summary

Background Alterations in specific signal transduction pathways may explain the hyperproliferation and abnormal differentiation of the keratinocytes as well as the increased expression of inflammatory cytokines seen in psoriasis. Major signalling pathways used by eukaryotic cells to transduce extracellular signals into cellular responses impinge on the mitogen-activated protein kinases (MAPKs).
Objectives To investigate the expression of the MAPK p38, extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) in psoriatic skin.
Methods Keratome biopsies were taken from patients with plaque-type psoriasis. Western blot analysis was used to determine p38, ERK and JNK activity and protein levels, whereas kinase assays were used to examine the kinase activity of p38.
Results We demonstrated increased levels of the phosphorylated forms of p38 and ERK1/2 in lesional psoriatic skin compared with nonlesional psoriatic skin. No abnormality was found in the activation and expression of JNK1/2. *Ex vivo* kinase assays confirmed the increased activation of p38, and furthermore demonstrated increased kinase activity of the p38 isoforms p38α, p38β and p38δ in lesional compared with nonlesional psoriatic skin. p38γ was not detected in the psoriatic skin. Clearance of the psoriatic lesions, induced by climatotherapy at the Dead Sea for 4 weeks, led to a normalization in the activity of both p38 and ERK1/2.
Conclusions Taken together, our results demonstrate that the activity of the MAPKs p38α, p38β and p38δ and ERK1/2 are increased in lesional psoriatic skin compared with nonlesional psoriatic skin, and that clearance of psoriasis normalizes the p38 and ERK1/2 activity. Thus, p38 and ERK1/2 might be potential targets in the treatment of psoriasis.

Psoriasis is a chronic inflammatory skin disorder affecting approximately 2% of the European population.[1] It is characterized by infiltration of the skin by activated T cells and an abnormal proliferation and differentiation of the keratinocytes. It has been suggested that psoriatic keratinocytes have abnormalities in expression and/or activation of different transcription factors as well as abnormalities in several signalling pathways.[2,3]

Regulatory mechanisms controlling proliferation, differentiation, immune responses and apoptosis of cells involve intracellular protein kinase cascades that can transduce signals from the cell's surface into changes in gene expression. The mitogen-activated protein kinase (MAPK) cascades are among the best characterized of these intracellular signalling pathways.[4,5] The MAPKs are serine–threonine kinases that, in response to a wide array of extracellular stimuli, are activated by phosphorylation on threonine and tyrosine residues. The MAPK pathway consists of a three-kinase module which includes a MAPK kinase kinase that activates a MAPK kinase which, in turn activates MAPK.[4] Activated MAPKs translocate to the nucleus where they phosphorylate a variety of transcription factors and other target proteins and thereby regulate the transcription of target genes.[6] Four different and distinctly regulated groups of MAPK have been described. These include the p38 MAPKs (p38α/β/δ/γ), the extracellular signal-regulated protein kinases (ERK1/2, also referred to as p44/p42 MAPKs), the Jun NH_2-terminal kinases (JNK1/2), and ERK5. The ERK pathway is predominantly activated by growth factors and other mitogens and has been shown to be involved in the vitamin D-induced differentiation of

keratinocytes[7] and Caco-2 cells.[8] In contrast, p38 and JNK are preferentially activated by inflammatory cytokines and environmental stresses such as osmotic shock and ultraviolet (UV) irradiation.[6,9] The p38 isoforms, p38α, p38β and p38δ, but not p38γ are expressed in the epidermis.[10,11] It has been suggested that p38α and p38β play important roles in mediating keratinocyte responses to cellular stress,[12] whereas p38δ has been shown to play an important role in inducing keratinocyte differentiation.[12]

The purpose of this study was to examine whether the expression and the activity of the MAPKs were altered in lesional psoriatic skin compared with nonlesional psoriatic skin. We demonstrated that the activity of the p38 isoforms, p38α, p38β and p38δ, are increased in involved psoriatic skin compared with noninvolved psoriatic skin, whereas their protein levels are not changed. Furthermore, clearance of psoriasis by climatotherapy at the Dead Sea for 4 weeks leads to a complete normalization of p38 and ERK1/2.

Materials and methods

Materials

Keratinocyte serum-free medium (KSFM) and keratinocyte basal medium (KBM) were obtained from Invitrogen (Carlsbad, CA, U.S.A.). p38 MAPK assays and antibodies against phospho-p38, p38α, p38δ, p38, phospho-MAPK (p44/p42), MAPK (p44/p42), phospho-JNK1/2, JNK1/2, activating transcriptional factor (ATF)-2 and phospho-ATF-2 were purchased from Cell Signalling Technology (Beverly, MA, U.S.A.). Anti-TBP (TATA binding protein), anti-p38β and anti-p38γ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase (HRP)-conjugated antirabbit IgG antibody was from DAKO (Glostrup, Denmark).

Biopsies

Keratome biopsies were obtained from lesional and nonlesional psoriatic skin as previously described.[3] Informed consent was obtained from each patient. The patients had untreated plaque-type psoriasis. Furthermore, biopsies were taken from lesional psoriatic skin before and after clearance. Clearance was induced by climatotherapy at the Dead Sea for 4 weeks.

Cell cultures

Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as previously described.[13] First-passage keratinocytes were grown in KSFM. Twenty-four hours before UVB stimulation (250 J m^{-2}) the medium was changed to KBM (the same as KSFM but without growth factors) in which the cells were stimulated. The UV source was a linear bank of UVB fluorescence tubes emitting UVB light (Phillips TL12). In some experiments the keratinocytes were pretreated with the p38α/β inhibitor SB 202190 (10 μmol L^{-1}) for 30 min before UVB stimulation. Following UVB stimulation the cells were incubated for 15 min before extraction. Cells were grown at 37 °C and 5% CO_2 in an incubator.

Western blot analysis

Total cell extracts were prepared from keratome biopsies taken from psoriatic patients using the TRIZOL reagent according to the recommendation of the manufacturer (Invitrogen). Equal amounts of protein (as determined by Bradford) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted on to nitrocellulose membranes.[14] Membranes were incubated with the appropriate primary antibody and detected with antirabbit IgG-HRP (DAKO) in a standard ECL reaction (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. Densitometric analysis of the band intensity was carried out using Kodak 1D Image Analysis Software.

In vitro kinase assays

The activities of the p38 MAPKs were measured using nonisotopic p38 MAPK assay systems (Cell Signalling Technology). Anti-p38α, anti-p38β and anti-p38δ MAPK antibodies (2 μg per sample) were adsorbed to Protein G-Sepharose beads for 2 h at 4 °C. p38α, p38β and p38δ were immunoprecipitated by incubating 200 μg of total cellular protein overnight at 4 °C with the adsorbed Sepharose beads. Immunocomplexes were isolated and washed three times with 0·5 mL ice-cold lysis buffer (20 mmol L^{-1} Tris pH 7·5, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} ethylenediamine tetraacetic acid, 1 mmol L^{-1} ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, 1% Triton X-100, 2·5 mmol L^{-1} sodium pyrophosphate, 1 mmol L^{-1} β-glycerol phosphate, 1 mmol L^{-1} Na_3VO_4, 1 μg mL^{-1} leupeptin and 1 mmol L^{-1} phenylmethylsulphonyl fluoride). Thereafter the immunocomplexes were washed twice with ice-cold kinase buffer (25 mmol L^{-1} Tris pH 7·5, 5 mmol L^{-1} β-glycerolphosphate, 2 mmol L^{-1} dithiothreitol, 0·1 mmol L^{-1} Na_3VO_4 and 10 mmol L^{-1} $MgCl_2$). Immunocomplexes were then incubated for 30 min at 30 °C with 50 μL kinase buffer supplemented with 200 μmol L^{-1} adenosine triphosphate and 2 μg ATF-2 fusion protein. The reaction was stopped by the addition of 25 μL 3 × SDS sample buffer and boiling for 5 min. Samples were centrifuged for 2 min (12 000 **g**), and 30 μL of the supernatant was then resolved by SDS–PAGE (10–20% gradient gel) and electroblotted on to a nitrocellulose membrane.

Statistical analysis

For statistical analysis a Student's t-test was done. To test for normal distribution, a probability test was made. $P < 0·05$ was regarded as statistically significant.

Results

Activity and protein levels of the mitogen-activated protein kinases p38, ERK1/2 and JNK1/2 in lesional psoriatic skin compared with nonlesional psoriatic skin

To investigate the protein levels and the activity of the MAPKs p38, ERK1/2 and JNK1/2 in psoriatic skin, whole cell extracts from lesional and nonlesional psoriatic skin were isolated. Western blotting experiments demonstrated that the level of the active phosphorylated form of p38 was significantly increased (4·3-fold as determined by densitometric analysis, $P < 0·05$) in cell extracts from lesional psoriatic skin compared with nonlesional psoriatic skin from the same patient. No significant changes were found in the total protein level of p38 (Fig. 1). In two of four patients investigated, active phosphorylated ERK1 and ERK2 were increased in lesional psoriatic skin compared with nonlesional psoriatic skin, whereas the protein levels of total ERK1 and ERK2 were unchanged (Fig. 1). Neither the protein levels of JNK1 and JNK2 nor the level of the active phosphorylated forms of JNK1 and JNK2 were significantly changed in lesional psoriatic skin compared with nonlesional skin. Equal protein loading was confirmed by assessing the protein level of TBP.

Activity and protein levels of the mitogen-activated protein kinases p38, ERK1/2 and JNK1/2 before and after clearance with climatotherapy

Biopsies were obtained from psoriatic patients before and after clearance by climatotherapy at the Dead Sea for 4 weeks. Whole cell extracts were isolated and analysed by Western blotting. It was found that the active phosphorylated forms of both p38 and ERK1/2 were significantly higher (2·8-fold, $P < 0·05$ and 2·1-fold, $P < 0·05$, respectively) in biopsies taken from untreated lesional psoriatic skin, compared with post-treatment biopsies (Fig. 2). No significant changes in the active phosphorylated forms of JNK1/2, together with the protein levels of p38, ERK1/2 and JNK1/2 were seen in the biopsies from pre- and post-treatment patients (Fig. 2). Equal protein loading was confirmed by assessing the protein level of TBP.

P38 kinase activity is increased in psoriatic skin

The above results showed an increased level of phosphorylated p38 in lesional psoriatic skin. To test if this was paralleled by an increased p38 kinase activity we performed a p38 kinase activity assay. As shown in Figure 3 the p38 kinase activity is significantly increased in lesional psoriatic skin compared with nonlesional psoriatic skin as measured by ATF-2 phosphorylation (6·9-fold, $P < 0·05$) (Fig. 3). Equal

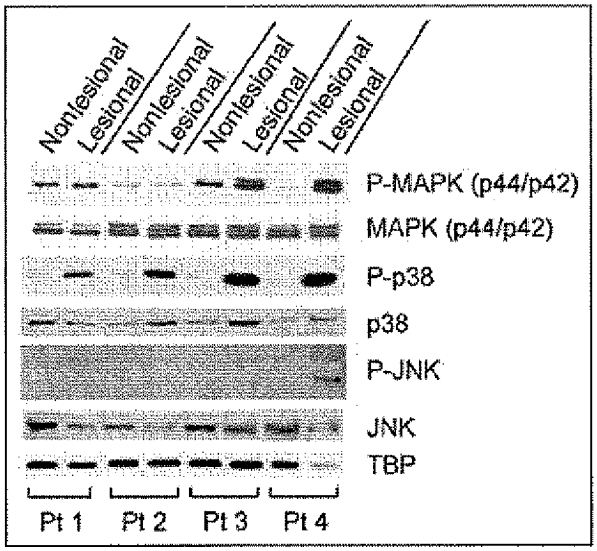

Fig 1. Mitogen-activated protein kinase (MAPK) expression in psoriatic skin. Whole cell protein extracts (50 μg of protein) were prepared from lesional and nonlesional psoriatic skin from patients with psoriasis vulgaris. The protein extracts were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on a 10–20% gradient gel. After electroblotting, the separated proteins were probed with the indicated antibodies. Equal loading was confirmed by incubation with an anti-TBP (TATA binding protein) antibody.

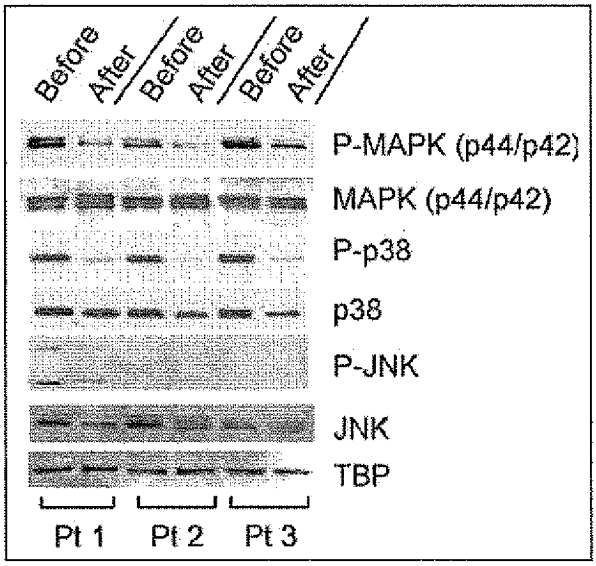

Fig 2. Clearance of the psoriatic patients leads to a normalization of the p38 and ERK1/2 activity. Biopsies were taken from psoriatic patients before and after clearance by climatotherapy at the Dead Sea for 4 weeks and whole cell protein extracts were prepared. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on a 10–20% gradient gel and the separated proteins were probed with the indicated antibodies. Equal loading was confirmed by incubation with an anti-TBP (TATA binding protein) antibody.

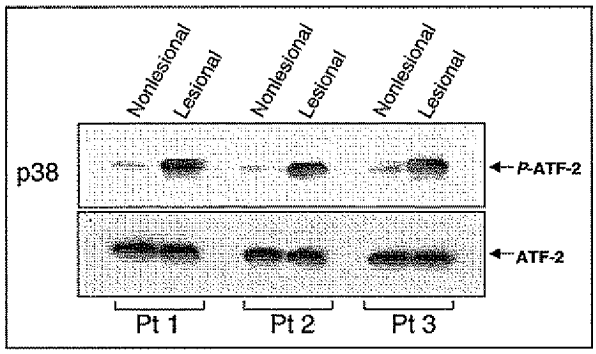

Fig 3. p38 kinase activity is increased in psoriatic skin. Whole cell protein extracts (200 μg of protein) from lesional and nonlesional psoriatic skin were prepared for immunoprecipitation of p38 mitogen-activated protein kinase. Enzyme activities were determined by an *in vitro* kinase assay using activating transcriptional factor (ATF)-2 as substrate. Kinase reactions were prepared for sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted with antiphospho-ATF-2 antibody. Equal loading was confirmed by incubation with an anti-ATF-2 antibody.

protein loading was determined by loading equal volumes of the immunocomplexed suspension and assessing the ATF-2 protein level.

The kinase activity of the p38 isoforms p38α, p38β and p38δ is increased in psoriatic skin

To determine which p38 isoforms are activated in psoriatic skin, we adsorbed anti-p38α, anti-p38β and anti-p38δ-specific antibodies to different Protein G-Sepharose beads and challenged these with extracts of lesional and nonlesional psoriatic skin. The immunocomplexes were then assayed for the ability to phosphorylate ATF-2. The kinase activities of p38α (2·1-fold, $P < 0{\cdot}05$), p38β (3·0-fold, $P < 0{\cdot}05$) and p38δ (2·7-fold, $P < 0{\cdot}05$) were significantly increased in lesional psoriatic skin compared with nonlesional psoriatic skin based on the ability to phosphorylate ATF-2. In contrast, the total protein levels of p38α, p38β and p38δ were not altered in lesional psoriatic skin vs. nonlesional psoriatic skin (Fig. 4a). The p38γ isoform could not be detected in keratome biopsies from psoriatic patients (data not shown).

In control experiments cultured normal human keratinocytes were pretreated with or without the p38 inhibitor SB 202190 (10 μmol L^{-1}) (known to selectively inhibit the p38α and p38β isoforms[15]) for 30 min before being stimulated with UVB (250 J m^{-2}). Whole cell extracts were isolated and a p38 kinase activity assay performed. SB 202190 (10 μmol L^{-1}) inhibited UVB-induced p38α and p38β kinase activity, but not p38δ kinase activity, indicating specificity of the antibodies and absence of cross-reactivity (Fig. 4b).

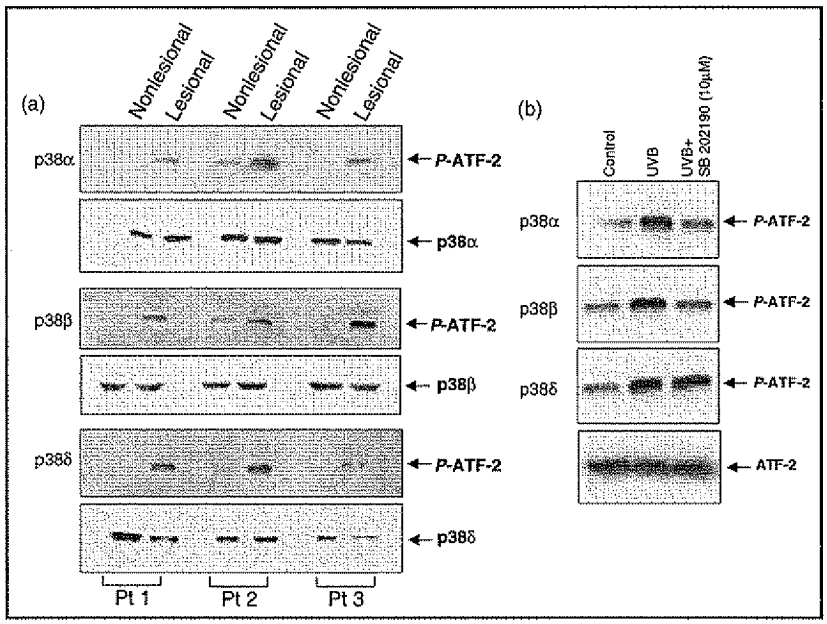

Fig 4. The kinase activity of the p38 isoforms p38α, p38β and p38δ are increased in psoriatic skin. (a) Equal amounts of protein (200 μg) from lesional and nonlesional psoriatic skin were precipitated using anti-p38α, anti-p38β or anti-p38δ antibodies and protein G-Sepharose beads. Activities of the precipitated kinases were monitored based on the ability to phosphorylate activating transcriptional factor (ATF)-2 fusion protein using an antibody specific for phosphor-ATF-2. p38α, p38β and p38δ protein levels were assayed by immunoblot using anti-p38α, anti-p38β and anti-p38δ antibodies. (b) Whole cell extracts were isolated from cultured human keratinocytes pretreated with or without the p38 inhibitor SB 202190 (10 μmol L^{-1}) for 30 min before ultraviolet B irradiation (250 J m^{-2}); 200 μg of protein were precipitated using anti-p38α, anti-p38β or anti-p38δ antibodies and protein G-Sepharose beads. A kinase reaction was performed and prepared for sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted with antiphospho-ATF-2 antibody. Equal loading was confirmed by incubation with an anti-ATF-2 antibody.

Discussion

The MAPKs control a wide variety of cellular events from very complex cellular programmes such as cell differentiation, cell proliferation and apoptosis to processes involved in immune responses. In the present study we have demonstrated that the activity of p38 and ERK1/2 are increased in lesional psoriatic skin compared with nonlesional psoriatic skin. By examining the different p38 isoforms we found that the kinase activity of p38α, p38β and p38δ were increased in lesional compared with nonlesional psoriatic skin. Furthermore, we showed that clearance of the psoriatic patients with climatotherapy at the Dead Sea normalizes the activity of p38 and ERK1/2.

In accordance with our results, previous studies have reported increased activation of ERK in lesional psoriatic skin.[16,17] Takahashi *et al.* found an increased level of the activated form of ERK1/2 as well as an increased activation of JNK in psoriatic skin. Furthermore, they found no difference in the activation of p38 between lesional and nonlesional psoriatic skin.[16] In contrast, our study revealed that not only was the phosphorylated form of p38 significantly increased in psoriatic skin, but also the p38 kinase activity was significantly increased in lesional vs. nonlesional psoriatic skin. We cannot explain the difference between the results obtained by Takahashi *et al.* and our results. However, different methods of collecting the skin samples have been used. Furthermore, we have determined p38 kinase activity that was not analysed by Takahashi *et al.*

We have shown that the p38 isoforms p38α, p38β and p38δ are all expressed, but that their expression is not regulated in psoriatic skin. p38γ was undetectable in the skin; this has also been found by others.[10] The kinase activity of p38α, p38β and p38δ was clearly augmented in lesional psoriatic skin compared with nonlesional psoriatic skin. Similar to other cellular systems, p38α and p38β have been found to be activated in keratinocytes treated with UV light, H_2O_2, tumour necrosis factor (TNF)-α and interleukin (IL)-1β, suggesting an important role of these two isoforms in mediating keratinocyte responses to cellular stress.[18,19] Because the keratinocytes in lesional psoriatic skin are characterized, not only by abnormal growth, but also by increased expression of inflammatory cytokines such as TNF-α and IL-8,[20,21] it is possible that the increased activity of p38α and p38β plays an important role in the inflammatory part of psoriasis. Interestingly, we found that the kinase activity of the p38δ isoform was upregulated in psoriatic skin. p38δ has previously been shown to play an important role in inducing keratinocyte differentiation,[12] and recent studies strongly suggested p38δ as the major p38 isoform driving the expression of the keratinocyte differentiation marker involucrin.[22,23] It is therefore of interest that the differentiation marker involucrin has been demonstrated to be increased in lesional psoriatic skin.[24] Thus, the increased kinase activity of p38δ seen in psoriatic skin might play a role in the psoriatic differentiation pattern by increasing involucrin expression.

The beneficial effect of climatotherapy at the Dead Sea for psoriatic patients is well known and has been used successfully for the treatment of moderate to severe psoriasis.[25–27] However, very little is known about the *in vivo* effect of climatotherapy at the Dead Sea at the molecular and cellular levels. Our data demonstrate for the first time a possible molecular explanation for the beneficial effect of climatotherapy at the Dead Sea.

In this study we have demonstrated that clinical expression of psoriasis is associated with increased p38 and ERK1/2 activity, suggesting that p38 and ERK1/2 might play a role in the pathophysiology of psoriasis. Because the expression and actions of important cytokines such as TNF-α are dependent on p38 and ERK1/2 activity, these MAPKs might be novel targets in the treatment of psoriasis.

Acknowledgments

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References

1 Koo J. Population-based epidemiologic study of psoriasis with emphasis on quality of life assessment. *Dermatol Clin* 1996; **14**:485–96.
2 McKenzie RC, Sabin E. Aberrant signalling and transcription factor activation as an explanation for the defective growth control and differentiation of keratinocytes in psoriasis: a hypothesis. *Exp Dermatol* 2003; **12**:337–45.
3 Johansen C, Kragballe K, Rasmussen M *et al.* The AP-1 DNA binding activity is decreased in lesional psoriatic skin compared to nonlesional psoriatic skin. *Br J Dermatol* 2004; **151**:600–7.
4 Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 1997; **9**:180–6.
5 Davis RJ. Transcriptional regulation by MAP kinases. *Mol Reprod Dev* 1995; **42**:459–67.
6 Karin M, Hunter T. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr Biol* 1995; **5**:747–57.
7 Johansen C, Kragballe K, Henningsen J *et al.* 1,25(OH)2D3 stimulates AP-1 DNA binding activity by a PI3-kinase/Ras/MEK/ERK1/2 and JNK1 dependent increase in c-Fos, Fra1 and c-Jun expression in human keratinocytes. *J Invest Dermatol* 2003; **120**:561–70.
8 Chen A, Davis BH, Bissonnette M *et al.* 1,25-Dihydroxyvitamin D(3) stimulates activator protein-1-dependent caco-2 cell differentiation. *J Biol Chem* 1999; **274**:35505–13.
9 Davis RJ. MAPKs: new JNK expands the group. *Trends Biochem Sci* 1994; **19**:470–3.
10 Dashti SR, Efimova T, Eckert RL. MEK7-dependent activation of p38 MAP kinase in keratinocytes. *J Biol Chem* 2001; **276**:8059–63.
11 Ono K, Han J. The p38 signal transduction pathway: activation and function. *Cell Signal* 2000; **12**:1–13.
12 Eckert RL, Efimova T, Balasubramanian S *et al.* p38 mitogen-activated protein kinases on the body surface—a function for p38 delta. *J Invest Dermatol* 2003; **120**:823–8.
13 Kragballe K, Desjarlais L, Marcelo CL. Increased DNA synthesis of uninvolved psoriatic epidermis is maintained *in vitro*. *Br J Dermatol* 1985; **112**:263–70.

14 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**:248–54.

15 Kumar S, McDonnell PC, Gum RJ *et al.* Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem Biophys Res Commun* 1997; **235**:533–8.

16 Takahashi H, Ibe M, Nakamura S *et al.* Extracellular regulated kinase and c-Jun N-terminal kinase are activated in psoriatic involved epidermis. *J Dermatol Sci* 2002; **30**:94–9.

17 Haase I, Hobbs RM, Romero MR *et al.* A role for mitogen-activated protein kinase activation by integrins in the pathogenesis of psoriasis. *J Clin Invest* 2001; **108**:527–36.

18 Zhang QS, Maddock DA, Chen JP *et al.* Cytokine-induced p38 activation feedback regulates the prolonged activation of AKT cell survival pathway initiated by reactive oxygen species in response to UV irradiation in human keratinocytes. *Int J Oncol* 2001; **19**:1057–61.

19 Garmyn M, Mammone T, Pupe A *et al.* Human keratinocytes respond to osmotic stress by p38 map kinase regulated induction of HSP70 and HSP27. *J Invest Dermatol* 2001; **117**:1290–5.

20 Ettehadi P, Greaves MW, Wallach D *et al.* Elevated tumour necrosis factor-alpha (TNF-alpha) biological activity in psoriatic skin lesions. *Clin Exp Immunol* 1994; **96**:146–51.

21 Jiang WY, Chattedee AD, Raychaudhuri SP *et al.* Mast cell density and IL-8 expression in nonlesional and lesional psoriatic skin. *Int J Dermatol* 2001; **40**:699–703.

22 Balasubramanian S, Efimova T, Eckert RL. Green tea polyphenol stimulates a Ras, MEKK1, MEK3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. *J Biol Chem* 2002; **277**:1828–36.

23 Efimova T, Broome AM, Eckert RL. A regulatory role for p38 delta MAPK in keratinocyte differentiation. Evidence for p38 delta-ERK1/2 complex formation. *J Biol Chem* 2003; **278**:34277–85.

24 Ishida-Yamamoto A, Iizuka H. Differences in involucrin immunolabeling within cornified cell envelopes in normal and psoriatic epidermis. *J Invest Dermatol* 1995; **104**:391–5.

25 Abels DJ, Kattan-Byron J. Psoriasis treatment at the Dead Sea: a natural selective ultraviolet phototherapy. *J Am Acad Dermatol* 1985; **12**:639–43.

26 Hodak E, Gottlieb AB, Segal T *et al.* Climatotherapy at the Dead Sea is a remittive therapy for psoriasis: combined effects on epidermal and immunologic activation. *J Am Acad Dermatol* 2003; **49**:451–7.

27 Harari M, Shani J. Demographic evaluation of successful antipsoriatic climatotherapy at the Dead Sea (Israel) DMZ Clinic. *Int J Dermatol* 1997; **36**:304–8.

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Expression and localization of the activated mitogen-activated protein kinase in lesional psoriatic skin

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Abstract

Abnormalities in several signaling pathways and in the expression and/or activation of different transcription factors in psoriatic keratinocytes have been hypothesized to play a role in the pathophysiology of psoriasis. The mitogen-activated protein kinase (MAPK) cascades are among the best characterized of intracellular signaling pathways, and they play important roles in cell proliferation, differentiation, gene expression, and inflammation. We investigated the expression, activation and distribution of extracellular signal-regulated kinases (ERKs), p38 mitogen-activated protein kinases (p38 MAPK) and c-Jun N-terminal kinases (JNKs), using immunohistochemistry and Western blot in lesional psoriatic skin and normal control skin, to clarify the possible roles of these kinases involved in the pathogenesis of psoriasis. The immunoblot analysis demonstrated that activation of ERK1/2 and p38 MAPK increased in the lesional psoriatic skin. In addition, a significant increase in p-MEK (the upstream activator of ERK), and p-CREB (a downstream transcription factor of active ERK) was also found in our experiment. The immunohistochemical study showed that the levels of phosphorylated ERK1/2 and p38 MAPK were enhanced in lesional psoriatic skin compared with controls. Phosphorylated ERK1/2 and p38 exhibited clear nuclear localization throughout the epidermal part of lesional psoriatic skin. These findings suggested that ERK1/2 and p38 pathways were involved in the pathophysiology of psoriasis.

Keywords: Extracellular signal-regulated kinase; p38 mitogen-activated protein kinase; c-Jun N-terminal kinase; Psoriasis

Introduction

Psoriasis is a common skin disorder, and estimates of its prevalence vary from 0.5% to 4.6% the world population. It is characterized by epidermal hyperplasia, increase in keratin expression, and recruitment of T cells as well as changes in the endothelial vascular system (Bos et al., 2005; Bowcock and Krueger, 2005; Krueger and Ellis, 2005). The dysfunction of immune system was known to be an important factor in the pathogenesis of psoriasis (Bowcock and Krueger, 2005), meanwhile strong evidence also indicated that keratinocytes contributed to the disease (Kwon et al., 2004; Park et al., 2005; Yu et al., 2006). Intracellular protein kinase cascades transduce signals from the cell surface into nucleus. Thereby, they contribute to the regulatory mechanism controlling cell proliferation, differentiation, and apoptosis. Abnormalities in several signaling pathways and in the expression or activation of different transcription factors in psoriatic keratinocytes have been hypothesized to play a role in the pathophysiology of psoriasis (McKenzie and Sabin, 2003).

The mitogen-activated protein kinase (MAPK) cascades are a group of important pathways among these intracellular protein kinase cascades (Robinson and Cobb, 1997). Three main MAPK have been well characterized: the extracellular signal-regulated protein kinase 1/2 (ERK1/2), the p38 mitogen-activated protein kinases (p38 MAPKs) and the c-Jun N-terminal kinase (JNKs). Upon activation by phosphorylation of both threonine and tyrosine residues, these kinases phosphorylate intracellular

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enzymes and transcription factors. ERK can be activated in response to growth factor (Boulton et al., 1991), oxidative stress (Aikawa et al., 1997), and increases of intracellular calcium levels or glutamate receptor stimulation (Robinson and Cobb, 1997). Phosphorylation of ERK links to immediate early gene induction, and hyperphosphorylation of the nuclear transcription factor E-26-like protein 1 (Elk-1) and cAMP/calcium-responsive element binding protein (CREB) (Sgambato et al., 1998). p38 and JNK are activated by stress signals such as inflammatory cytokinase, heat shock, ultraviolet light and ischemia (Kyriakis and Avruch, 1996). Phosphorylation of p38 can active MAPK-activated protein 2 and transcription factor (ATF) 2, and phosphorylated JNK can activate c-Jun directly (Van Dam et al., 1995; Derijard et al., 1995).

The importance of MAPKs as pathological modulators is being increasingly recognized (Mielke and Herdegen, 2000). In the present study, we hypothesized that there would be some alterations on MAPK expression and activation between lesional psoriatic skin and normal control skin.

Materials and methods

Tissue samples

We obtained skin biopsies of psoriatic lesions from 24 outpatients (mean age=39.7 years; skin involvement 15–40%) at Shandong University Hospital. The patients had untreated plaque-type psoriasis and the biopsies were taken from the center of a plaque with moderate-to-severe psoriasis from either the upper or lower extremities. We obtained normal skin from 10 anatomic-matched healthy individuals (mean age=42.2 years). Informed consent was obtained from all subjects before the study. The medical ethical committee of Shandong approved the study.

Antibodies

Mouse antiphospho-ERK1/2 (p-ERK1/2) antibody, rabbit anti-ERK1/2 antibody, mouse antiphospho-p38 (p-p38) antibody, mouse anti-p38 antibody, mouse antiphospho-JNK (p-JNK) antibody, mouse anti-JNK antibody and rabbit anti-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antiphospho -MEK (p-MEK) and mouse antiphospho-CREB (p-CREB) were obtained from Upstate (NY, USA).

Immunohistochemistry

Tissue specimens were fixed in 10% formalin, embedded in paraffin and sectioned. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in 60% methanol for 15 min. The sections were blocked with 5% normal goat serum to prevent non-specific staining by the secondary antibody, and then were incubated overnight at 4 °C with each primary antibody. After the sections were washed, they were incubated with the secondary antibody conjugated with horseradish peroxidase at a 1:400 dilution for 1 h at room temperature. After several washed, color was developed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB) used as a cosubstrate for 5–7 min. Negative controls sections were treated identically except for the absent of primary antibody.

Western blot analysis

The skin was incubated with 1000 U/ml dispase in phosphate-buffered saline for overnight at 4 °C. Following this, the epidermis was separated from the dermis. The epidermis was homogenized in ice-cold lysis buffer (20 nM Tris–Base, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and left on ice for 30 min. The samples were centrifuged at 10,000×g for 10 min at 4 °C. Equal amounts of protein were boiled for 5 min and electrophoresed on a 12% SDS–polyacrylamide gel (PAGE) for 45 min at 200 V. Proteins were transferred to a 0.22 μm nitrocellulose membrane (BioRad, Hercules, CA) at 30 V for 1 h. Following blocking with 5% non-fat milk, the blots were washed with PBS containing 0.1% Tween-20, and then were incubated at 4 °C for overnight with primary antibodies were dilated 1:500 in PBS. Anti-β-actin antibody was used at 1:1000. After several washes, the membranes were incubated at room temperature for 1 h with the appropriate secondary antibody conjugated to HRP, and then washed again. The blots were then visualized with enhanced chemiluminescence (ECL) by film.

Statistical analysis

The results are expressed as mean±SD. Statistical analysis was performed using Student's *t*-test. $p<0.05$ was considered statistically significant.

Results

Distribution of phosphorylated ERK1/2, p38 MAPK, JNK in the lesional psoriatic skin and normal human skin

Immunohistochemistry was performed using phosphorylation site-specific antibodies to p-ERK1/2, p-p38 and p-JNK, to determine the distribution of the activated MAPKs in lesional psoriatic skin compared with normal control skin (Fig. 1). Immunohistochemistry revealed enhanced phosphorylation of ERK1/2 and p38 in psoriatic epidermis. In the keratinocytes of psoriatic epidermis, p-ERK1/2 expressed mainly in nucleus, partially in cytoplasms, and about 96% (n=23 of 24) psoriatic lesions examined showed this pattern. Similarly, strong staining for p-p38 was observed in both nucleus and cytoplasms in all psoriatic epidermal layers, and such pattern account 83% (n=19 of 24) in all. In normal skin, p-ERK1/2 and p-p38 exhibited a weaker, diffuse cytoplasmic staining in most epidermal keratinocytes, with little or absent nucleus staining. Different with p-ERK1/2 and p-p38, p-JNK expressed weekly in cytoplasm of keratinocytes, and the staining intensity was comparable between two groups.

Upregulation of phosphorylated ERK1/2 and p38 in the lesional psoriatic skin

To determine whether the MAPK cascade was altered in psoriatic lesions, whole epidermis extracts from psoriatic lesions and anatomic site-matched control skin, were subject to Western blot using antibodies against p-ERK1/2, p-p38, and p-JNK. Moreover, MEK and CREB were also detected, which were the upstream and downstream signal molecules of ERK1/2, respectively.

Compared to normal control skin, increased p-ERK1/2 level was detected by Western blot analysis in lesional psoriatic epidermis, whereas the level of total ERK1/2 remained comparable between normal versus psoriatic epidermis. As the upstream physiological activator of ERK1/2, p-MEK expressed higher in psoriatic lesions compared to normal control skin. With regard to p-CREB, there was also a sig-

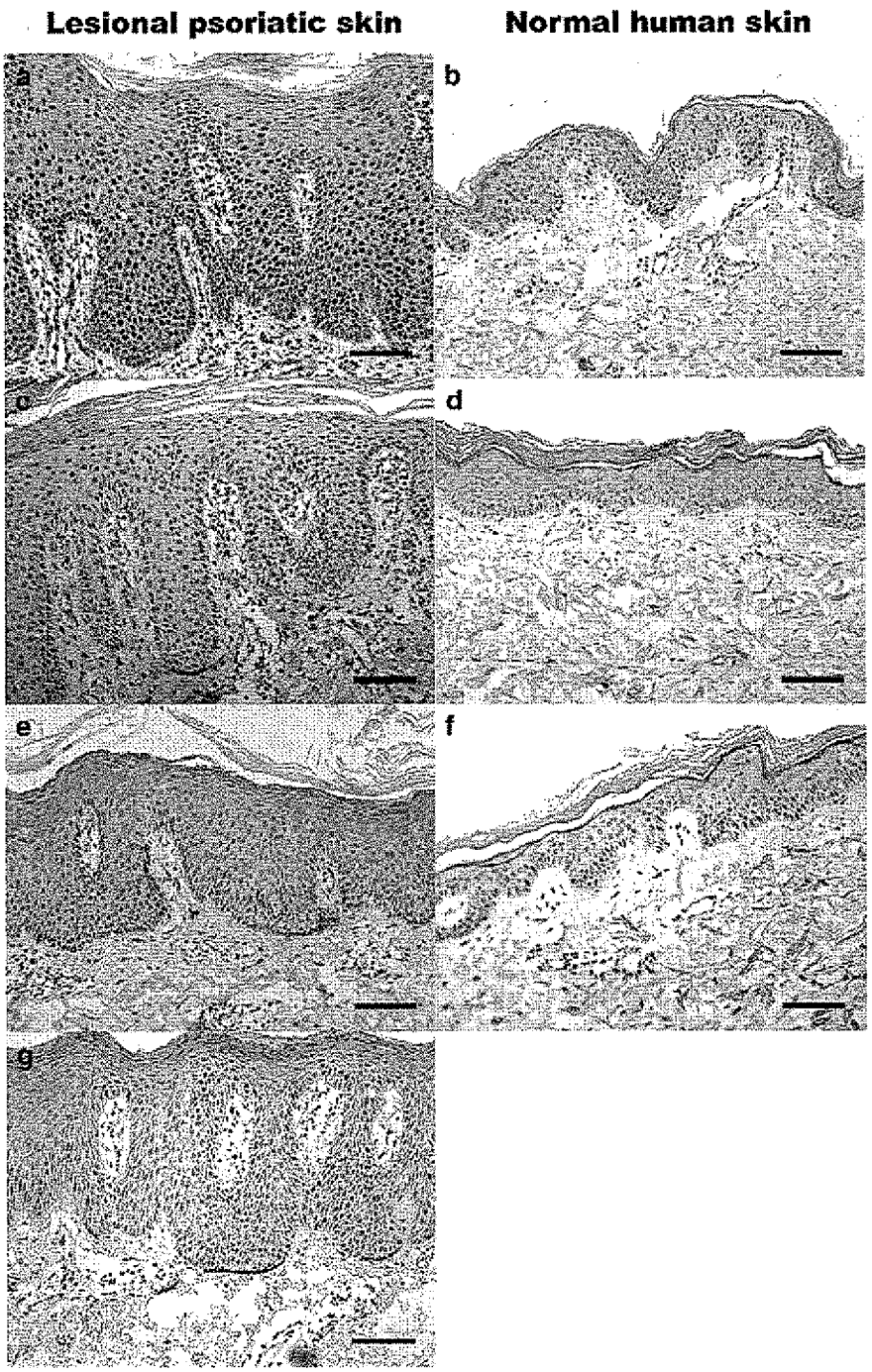

Fig. 1. Representative results of the phosphorylation of ERK1/2 (a, b), p-p38 (c, d), and p-JNK (e, f) in psoriatic versus normal skin samples. In human psoriatic lesion, the hyperplasic epidermis showed an increased level of p-ERK1/2 (a) and p-p38 (c), particularly in the nuclei of keratinocytes as compared with that in normal human epidermis (b, d). Weak cytoplasmic staining for p-JNK was observed in the psoriatic lesion (e) and normal epidermis (f). IgG negative controls were uniformly unstained (g) and positive controls were mammary cancer (data not show). Scale bars: 100 μm; original magnification: 200×.

nificant increase in psoriatic skin. These results demonstrated the activation of ERK cascade in the lesion of psoriasis (Fig. 2).

A similar increase in p-p38 was detected in psoriatic epidermis compared to normal control skin, although the level of total p38 remained unchanged (Fig. 3). In terms of p-JNK and total JNK, there was no significant difference between normal versus psoriatic epidermis.

Discussion

The mitogen-activated protein kinase (MAPK) represents important regulatory signaling molecules that serve as integration points connecting extracellular signals to the transcriptional programs of the cell (Robinson and Cobb, 1997). MAPK is activated in the cytoplasm and then translocated to the nucleus,

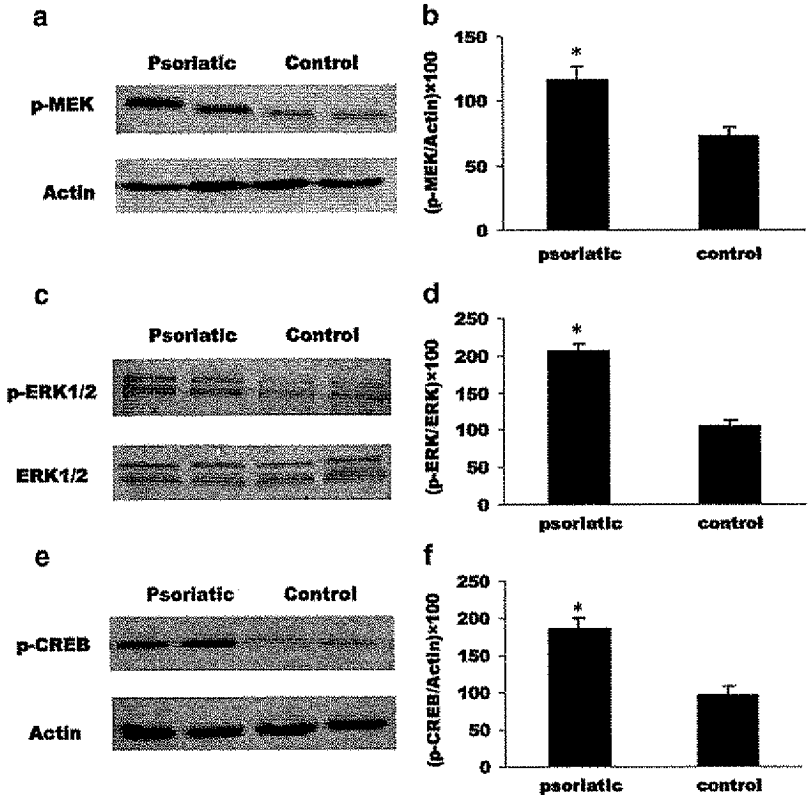

Fig. 2. Activation of ERK cascade. Immunoblot analysis of (a) p-MEK protein, (c) p-ERK, and (e) p-CREB in the lesional psoriatic skin and normal human skin. The immunoblot was quantified by scanning densitometry. The densities of p-MEK (b) and p-CREB (f) were normalized with those of actin, respectively; and the density of p-ERK1/2 (b) was normalized with total-ERK. $n=18$ for psoriatic group and $n=10$ for normal human group. $*p<0.01$ compared with the control group as assessed by Student's t-test.

where it induces gene expression and promotes cell cycle entry (Kim-Kaneyama et al., 2000; Zheng and Guan, 1994). The use of highly specific antibodies that recognize activated signaling molecules within the cellular context allows the analysis of potential relationships between intracellular phosphorylation mechanisms and diseases. With this approach, we can observe perturbations in a particular intracellular pathway that may lead to the abnormal pathophysiology of psoriasis.

The present study showed that p-ERK1/2 and p-p38 widely expressed in the lesional psoriatic epidermis, and exhibited a clear nuclear localization throughout the entire epidermal part of lesional psoriatic skin. In contrast, in normal skin, p-ERK1/2 and p-p38 predominantly stained in the cytoplasmic region of epidermal keratinocytes with absent or little nuclear stain. Previous studies also observed activation of ERK in basal and suprabasal keratinocytes of human and transgenic mouse psoriatic lesions (Haase et al., 2001). In addition, they found keratinocyte ERK could be activated by ligation of suprabasal integrins or treatment with IL-1α. The phosphorylated of ERK and p38 were known to translocate to the nucleus where they phosphorylated the transcription factors, which activated genes and increased the expression of corresponding protein products. Constitutive activation of MAPK increased the growth rate of human keratinocytes and delayed the onset of terminal dif-

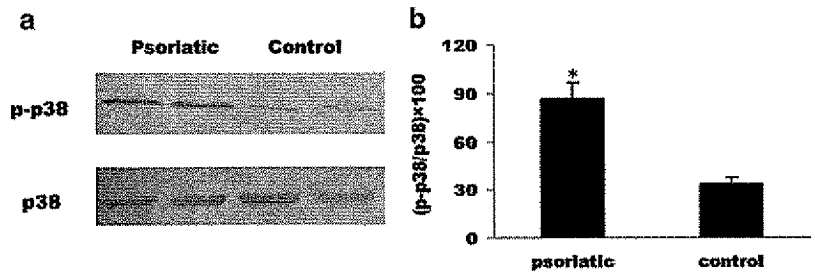

Fig. 3. Immunoblot analysis of p-p38 MAPK in the lesional psoriatic skin and normal human skin (a). The immunoblot was quantified by scanning densitometry. The density of p-p38 MAPK was normalized with total-p38 MAPK (b). p-p38 MAPK intensity showed a significant increase in the lesional psoriatic skin. Data are expressed as mean±SD; $n=18$ for psoriatic group and $n=10$ for normal human group; $*p<0.01$ compared with the control group as assessed by Student's t-test.

ferentiation, recreating many of the histological features of psoriatic epidermis.

Analysis of psoriatic skin showed that the levels of both ERK1/2 and p38 activity were increased, which was consistent with previous studies (Takahashi et al., 2002; Johansen et al., 2005). Meanwhile, the p-MEK and p-CREB were also found increased in lesional psoriatic skin, which indicated the whole ERK1/2 pathway was activated. Phosphorylated ERK translocates to the nucleus where it phosphorylates the transcription factors such as CREB, which activated genes implicated in cell proliferation. Several studies indicated the connection between the activation of CREB and induction of IL-6, IL-8, and TNF-α. CREB-binding sites were found in the promoter regions of TNF-α (Kuprash et al., 1999; Chong et al., 2003). IL-6 and IL-8 production was also found to be dependent on CREB phosphorylation (Ikewaki and Inoko, 2002; Persson et al., 2005). The keratinocytes in the lesional psoriatic skin are characterized, not only by abnormal proliferation and differentiation, but also by increased expression of inflammatory cytokines (Jiang et al., 2001; Ettehadi et al., 1994). On the basis of the analysis of the cytokines produced, psoriasis is often considered to be a T-helper 1 condition, as levels of TNF, IFN-γ, IL-1, IL-2, IL-6, and IL-8 are increased in psoriatic lesions (Bos et al., 2005; Bowcock and Krueger, 2005; Krueger and Ellis, 2005). Anti-TNF therapies have also been proved effective at treating psoriasis, demonstrating a critical role for this cytokine in the condition (Bos et al., 2005; Bowcock and Krueger, 2005; Krueger and Ellis, 2005). In addition, the ERK signaling pathway was associated with epidermal growth factor receptor (King et al., 1990), which was postulated to regulate the proliferation and differentiation of keratinocytes.

In addition, a strong link has been established between the p38 pathway and inflammation. The activation of the p38 pathway plays essential roles in the production of proinflammatory cytokines (IL-1, TNF-α, and IL-6) (Guan et al., 1998). Recent studies demonstrated increased kinase activity of the p38 isoforms p38α, p38β and p38δ in lesional psoriatic skin (Johansen et al., 2005). The participation of p38α in cell growth has been observed in mammals and treatment on mammalian cells with p38α/β inhibitor SB 203580 slowed proliferation as well (Takenaka et al., 1998). p38δ has previously been shown to play an important role in inducing keratinocyte differentiation (Eckert et al., 2003), and recent studies strongly suggested p38δ as the major p38 isoform driving the expression of the keratinocyte differentiation maker involucrin (Balasubramanian et al., 2002; Efimova et al., 2003). It is therefore of interest that the differentiation marker involucrin has been demonstrated to be increased in lesional psoriatic skin (Ishida-Yamamoto and Iizuka, 1995). Thus, the increased kinase activity of p38 seen in psoriatic skin might play a role in the psoriatic proliferation, differentiation, and inflammatory pattern.

In the present study, the phosphorylation of ERK and p38 was found increased in psoriatic lesions compared with normal control, which indicated that ERK and p38 might play a role in the pathophysiology of psoriasis. ERK and p38 pathways were critical for the proliferation and differentiation of keratinocyte (Eckert et al., 2003; Efimova et al., 2003). Based on these findings, we propose ERK1/2 and p38 pathways might be a promising therapeutic target for treatment of psoriasis.

Acknowledgments

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References

Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y., Yazaki, Y., 1997. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. J. Clin. Invest. 100, 1813–1821.

Balasubramanian, S., Efimova, T., Eckert, R.L., 2002. Green tea polyphenol stimulates a Ras, MEKK1, MEK3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. J. Biol. Chem. 277, 1828–1836.

Bos, J.D., de Rie, M.A., Teunissen, M.B., Piskin, G., 2005. Psoriasis: dysregulation of innate immunity. Br. J. Dermatol. 152, 1098–1107.

Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., Yancopoulos, G.D., 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65, 663–675.

Bowcock, A.M., Krueger, J.G., 2005. Getting under the skin: the immunogenetics of psoriasis. Nat. Rev., Immunol. 5, 699–711.

Chong, Y.H., Shin, Y.J., Suh, Y.H., 2003. Cyclic AMP inhibition of tumor necrosis factor alpha production induced by amyloidogenic C-terminal peptide of Alzheimer's amyloid precursor protein in macrophages: involvement of multiple intracellular pathways and cyclic AMP response element binding protein. Mol. Pharmacol. 63, 690–698.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I.C.H., Han, J., Ulevitch, R.J., Davis, R.J., 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science 267, 682–685.

Eckert, R.L., Efimova, T., Balasubramanian, S., Crish, J.F., Bone, F., Dashti, S., 2003. p38 mitogen-activated protein kinases on the body surface – a function for p38 delta. J. Invest. Dermatol. 120, 823–828.

Efimova, T., Broome, A.M., Eckert, R.L., 2003. A regulatory role for p38 delta MAPK in keratinocyte differentiation. Evidence for p38 delta-ERK1/2 complex formation. J. Biol. Chem. 278, 34277–34285.

Ettehadi, P., Greaves, M.W., Wallach, D., Aderka, D., Camp, R.D., 1994. Elevated tumour necrosis factor-alpha (TNF-alpha) biological activity in psoriatic skin lesions. Clin. Exp. Immunol. 96, 146–151.

Guan, Z., Buckman, S.Y., Pentland, A.P., Templeton, D.J., Morrison, A.R., 1998. Induction of cyclooxygenase-2 by the activated MEKK1→SEK1/MKK4→p38 mitogen-activated protein kinase pathway. J. Biol. Chem. 273, 2901–2908.

Haase, I., Hobbs, R.M., Romero, M.R., Broad, S., Watt, F.M., 2001. A role for mitogen-activated protein kinase activation by integrins in the pathogenesis of psoriasis. J. Clin. Invest. 108, 527–536.

Ikewaki, N., Inoko, H., 2002. A very late activating antigen-alpha 4 (CD49d) monoclonal antibody, BU49 induces phosphorylation of a cAMP response element-binding protein (CREB), resulting in induction of homotypic cell aggregation and enhancement of interleukin-8 (IL-8) production. Microbiol. Immunol. 46, 685–695.

Ishida-Yamamoto, A., Iizuka, H., 1995. Differences in involucrin immunolabeling within cornified cell envelopes in normal and psoriatic epidermis. J. Invest. Dermatol. 104, 391–395.

Jiang, W.Y., Chattedee, A.D., Raychaudhuri, S.P., Raychaudhuri, S.K., Farber, E.M., 2001. Mast cell density and IL-8 expression in nonlesional and lesional psoriatic skin. Int. J. Dermatol. 40, 699–703.

Johansen, C., Kragballe, K., Westergaard, M., Henningsen, J., Kristiansen, K., Iversen, L., 2005. The mitogen-activated protein kinases p38 and

ERK1/2 are increased in lesional psoriatic skin. Br. J. Dermatol. 152, 37–42.

Kim-Kaneyama, Nose, K., Shibanuma, M., 2000. Significance of nuclear relocalization of ERK1/2 in reactivation of c-*fos* transcription and DNA synthesis in senescent fibroblasts. J. Biol. Chem. 275, 20685–20692.

King Jr., L.E., Gates, R.E., Stoscheck, C.M., Nanney, L.B., 1990. Epidermal growth factor/transforming growth factor alpha receptors and psoriasis. J. Invest. Dermatol. 95, 10S–12S.

Krueger, G., Ellis, C.N., 2005. Psoriasis – recent advances in understanding its pathogenesis and treatment. J. Am. Acad. Dermatol. 53, S94–S100.

Kuprash, D.V., Udalova, I.A., Turetskaya, R.L., Kwiatkowski, D., Rice, N.R., Nedospasov, S.A., 1999. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. J. Immunol. 162, 4045–4052.

Kwon, Y.W., Kwon, K.S., Moon, H.E., Park, J.A., Choi, K.S., Kim, Y.S., Jang, H.S., Oh, C.K., Lee, Y.M., Kwon, Y.G., Lee, Y.S., Kim, K.W., 2004. Insulin-like growth factor-II regulates the expression of vascular endothelial growth factor by the human keratinocyte cell line HaCaT. J. Invest. Dermatol. 123, 152–158.

Kyriakis, J.M., Avruch, J., 1996. Protein kinase cascades activated by stress and inflammatory cytokines. BioEssays 18, 567–577.

McKenzie, R.C., Sabin, E., 2003. Aberrant signalling and transcription factor activation as an explanation for the defective growth control and differentiation of keratinocytes in psoriasis: a hypothesis. Exp. Dermatol. 12, 337–345.

Mielke, K., Herdegen, T., 2000. JNK and p38 stress kinases-degenerative effectors of signal-transduction-cascades in the nervous system. Prog. Neurobiol. 61, 45–60.

Park, H.J., Kim, H.J., Lee, J.H., Lee, J.Y., Cho, B.K., Kang, J.S., Kang, H., Yang, Y., Cho, D.H., 2005. Corticotropin-releasing hormone (CRH) downregulates interleukin-18 expression in human HaCaT keratinocytes by activation of p38 mitogen-activated protein kinase (MAPK) pathway. J. Invest. Dermatol. 124, 751–755.

Persson, E., Voznesensky, O.S., Huang, Y.F., Lerner, U.H., 2005. Increased expression of interleukin-6 by vasoactive intestinal peptide is associated with regulation of CREB, AP-1 and C/EBP, but not NF-kappa B, in mouse calvarial osteoblasts. Bone 37, 513–529.

Robinson, M.J., Cobb, M.H., 1997. Mitogen-activated protein kinase pathways. Curr. Opin. Cell Biol. 9, 180–186.

Sgambato, V., Pages, C., Rogard, M., Besson, M.J., Caboche, J., 1998. Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. J. Neurosci. 18, 8814–8825.

Takahashi, H., Ibe, M., Nakamura, S., Ishida-Yamamoto, A., Hashimoto, Y., Iizuka, H., 2002. Extracellular regulated kinase and c-Jun N-terminal kinase are activated in psoriatic involved epidermis. J. Dermatol. Sci. 30, 94–99.

Takenaka, K., Moriguchi, T., Nishida, E., 1998. Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. Science 280, 599–602.

Van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P., Angel, P., 1995. ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-*jun* induction in response to genotoxic agents. EMBO J. 14, 1798–1811.

Yu, X.J., Li, C.Y., Wang, K.Y., Dai, H.Y., 2006. Calcitonin gene-related peptide regulates the expression of vascular endothelial growth factor in human HaCaT keratinocytes by activation of ERK1/2 MAPK. Regul. Pept. 137, 134–139.

Zheng, C.F., Guan, K.L., 1994. Cytoplasmic localization of the mitogen-activated protein kinase activator MEK. J. Biol. Chem. 269, 19947–19952.

Increase in TNF-α and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and reduction with efalizumab (anti-CD11a)

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We find that CD11c$^+$ cells with many markers of dendritic cells (DCs) are a major cell type in the skin lesions of psoriasis. These CD11c$^+$ cells, which are evident in both epidermis and dermis, are the sites for the expression of two mediators of inflammation, inducible nitric oxide synthase (iNOS) and TNF-α in diseased skin. These cells express HLA-DR, CD40, and CD86, lack the Langerin and CD14 markers of Langerhans cells and monocytes, respectively, and to a significant extent express the DC maturation markers DC-LAMP and CD83. Treatment of psoriasis with efalizumab (anti-CD11a, Raptiva) strongly reduces infiltration by these DCs in patients responding to this agent. Disease activity after therapy was more related to DC infiltrates and iNOS mRNA levels than T cell infiltrates, and CD11c$^+$ cells responded more quickly to therapy than epidermal keratinocytes. Our results suggest that a type of DC, which resembles murine "Tip-DCs" that can accumulate during infection, has proinflammatory effects in psoriasis through nitric oxide and TNF-α production, and can be an important target for suppressive therapies.

autoimmune disease | CD11c | Tip-DC

The common skin disease psoriasis serves as an accessible, model type 1 autoimmune disease (1–3). Evidence for this view includes (*i*) a marked increase in the number of T cells in diseased skin, (*ii*) the differentiation characteristics of these T cells, including a skin-homing memory phenotype, a T helper 1/Tc1 predominance, and oligoclonality, and (*iii*) the disease improvements brought about by a variety of T cell-targeted immunosuppressive agents (4–6). T cell functions are often controlled by dendritic cells (DCs), and interestingly, psoriasis is characterized by the production of IL-23 by CD11c$^+$ DCs in lesional skin (7). IL-23 is known to be a pivotal cytokine for inflammation during experimental autoimmune encephalomyelitis in mice (8, 9).

To study the contribution and source of inflammatory molecules in the pathogenesis of psoriasis (2), we have engaged in detailed studies of disease resolution induced by specific biologic antagonists. We have emphasized inflammatory mediators associated with CD11c$^+$ cells, where high-level expression of this integrin is a marker for many types of DCs (10, 11). We now report that CD11c$^+$ cells lacking the monocyte marker CD14 are greatly increased in the dermis and epidermis of psoriasis lesions, such that CD11c$^+$ cells exceed T cells. Two critical mediators of inflammation, TNF and inducible nitric oxide synthase (iNOS), are simultaneously and primarily found in these CD11c$^+$ cells, which express HLA-DR, CD40 CD86, and to some extent, DC maturation markers DC-LAMP and CD83. Successful treatment of psoriasis with efalizumab (5), which targets CD11a on leukocytes, strongly reduces infiltration by these inflammatory DCs, which are similar to a cell type recently described in the mouse: the TNF- and iNOS-producing DC or "Tip-DC" (12–14). Given recent evidence that TNF inhibitors are able to reverse disease activity in psoriasis (15, 16), our data suggest that Tip-DCs are a major inflammatory and effector cell in psoriasis.

Materials and Methods

Skin Samples. Psoriasis lesions, nonlesional skin, and normal skin were obtained from patients and normal volunteers under an approved protocol (The Rockefeller University). Experiments were conducted on samples obtained from a previously published placebo-controlled clinical trial with efalizumab (n = 65) (Genentech and Xoma) (5). Patients treated with efalizumab showed greater clinical improvement and reduction of epidermal hyperplasia in skin lesions compared to placebo treatment. We consider that thin epidermis without keratin 16 (K16) expression represents histopathologic remission of psoriasis. None of the patients in the placebo group were K16$^-$ at day 56, whereas 29 patients (37%) in the treatment arm were K16$^-$ at the end of the study. Samples from other clinical trials with efalizumab (weekly 1 mg/kg s.c. for 12 weeks) were used to measure iNOS mRNA (n = 13, see Fig. 4*B*), and a time-course of CD11c$^+$ cell counts (n = 18, see Fig. 4*D*).

Antibodies. For immunohistochemistry, we used mouse anti-human monoclonal antibodies to K16 (Sigma), CD3 (Becton Dickinson), CD8 (BD PharMingen), CD83 (Becton Dickinson), CD1a (Becton Dickinson), CD11c (BD PharMingen), iNOS (R & D Systems) and CD14 (BD PharMingen). For immunofluorescence, we localized CD11c (BD PharMingen), DC-LAMP (Immunotech, Westbrook, ME), CD83 (Immunotech) (1:50–1:100) with appropriate IgG goat anti-mouse Ig conjugated to Alexa Fluor 488 or 546 (1:250) (Molecular Probes). The second primary antibodies for two-color labeling were conjugated to the fluorochrome or labeled by using the appropriate labeling kit (Molecular Probes) (1:100–1:500): iNOS (R & D Systems) Alexa Fluor 546, TNF-α FITC (Becton Dickinson), HLA-DR FITC (Becton Dickinson), HLA-DR phycoerythrin (PE) (Becton Dickinson), Langerin (Immunotech) Alexa Fluor 488. The TNF signal was amplified with a second secondary goat anti-FITC antibody (Molecular Probes) for 30 min at 1 μg/ml. Antibodies for FACS include the following: mouse IgG1 FITC (Becton

Conflict of interest statement: M.G. was employed by Xoma and W.D. was employed by Genentec at the time of the study.

Freely available online through the PNAS open access option.

Abbreviations: DC, dendritic cell; iNOS, inducible nitric oxide synthase; K16, keratin 16.

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Dickinson), mouse IgG1 PE (Becton Dickinson), mouse IgG1 peridinin-chlorophyll-protein (PerCP) (Becton Dickinson), HLA-DR allophycocyanin (Becton Dickinson), CD3 PerCP (Becton Dickinson), CD83 FITC (Immunotech), CD86 FITC (BD PharMingen), CD40 FITC (BD PharMingen), CD14 FITC (Becton Dickinson), and CD11c PE (Becton Dickinson).

Tissue Sections. Skin biopsies were frozen in optimal cutting temperature compound (Sakura Finetek, Tokyo), stored at −80°C, stained with hematoxylin (Fisher Scientific, Fair Lawn, NJ) and eosin (Shandon, Pittsburgh), or with mouse anti-human monoclonal antibodies as above using a published technique (17). Data for epidermal thickness and K16 staining are taken from all patients, whereas the rest of the antibodies in the panel (CD1a, CD11c, CD83, CD3, and CD8) were applied to 42 subjects in the active drug group and 26 subjects in the placebo group because of a limited number of tissue sections. For immunofluorescence, frozen lesional tissue sections from psoriasis patients ($n = 8$) were fixed in acetone and treated with 10% normal horse serum. Primary antibodies were incubated overnight at 4°C, the secondary antibody for 30 min, and the second primary antibody for 2 h. Images were acquired by using appropriate filters of a Zeiss Axioplan 2I microscope with Plan Apochromat 20 × 0.7 numerical aperture lens and a Hagamatsu orca ER-cooled charge-coupled device camera, controlled by METAVUE software (Universal Imaging). Alternatively, images were acquired by using appropriate filters of an upright confocal microscope with attached Zeiss 5 Fluar/0.25 and 10 Fluar/0.50 lenses controlled by least squares means analysis.

FACS. Skin shave biopsies were obtained from two psoriasis patients, and dermal cells allowed to emigrate in culture according to a published protocol (18). Shave biopsies are small superficial (split thickness) skin biopsies that give the largest surface area for efficient emigration of DCs. Briefly, the skin was cultured in dispase overnight, epidermis and dermis were separated, then dermis was cultured for 3 days, and the dermal supernatant was treated with collagenase for 1 h. FACS was performed with antibodies listed above, as described (19). A provisional DC gate (R1) was selected based on large cell size (FSC) and high side scatter (SCC) (see Fig. 2*E*) (20), and cells in this gate were further selected for CD11c expression (R2).

Analysis of iNOS Gene Expression. DCs were prepared and analyzed by quantitative PCR as described (6, 21). Monocytes or T cells were isolated by negative selection (Dynal, Oslo), and activated with CD3/CD28 beads (Dynal). HaCaT cells were grown in Dulbecco's modified eagle medium (R & D Systems).

Statistical Analysis. Significance was defined as $P < 0.05$ using a Student's t test. U scores and correlation coefficients were computed based on a recently published technique to analyze multivariate data (22).

Results

iNOS and TNF Double-Positive CD11c$^+$ Cells Are Greatly Increased in Psoriasis Lesions. Expression of iNOS mRNA is typically elevated by >10-fold in psoriasis lesions (4–6), and iNOS has been detected in psoriasis lesions (23). Because a product of iNOS, nitric oxide, is a key inflammatory mediator (24), we first set out to localize iNOS-producing cells in normal skin versus psoriasis lesions. Normal skin contained only a small number of iNOS$^+$ cells in the dermis (Fig. 1*A*), but abundant iNOS$^+$ cells were noted in both the epidermis and dermis of psoriasis lesions (Fig. 1*B*). In parallel, CD11c$^+$ cells had a similar distribution in both normal and diseased skin (Fig. 1 *C* and *D*). The iNOS$^+$ cells were large and stellate, with numerous dendritic processes (Fig. 5*A*, which is published as supporting information on the PNAS web site). Two-color immunofluorescence verified that iNOS was primarily localized to the CD11c$^+$ cells (Fig. 1*E*). Moreover, TNF was also expressed almost exclusively in these CD11c$^+$ cells (Fig. 1*F*), which failed to express the Langerhans cell markers, Langerin, (CD207) (Fig. 1*D*) or CD1a (data not shown), or the monocyte marker CD14 (Fig. 5*B*). These data indicate that the lesions of psoriasis contain numerous CD11c$^+$ cells that coexpress two key inflammatory mediators, iNOS and TNF, but are not typical Langerhans cells or monocytes.

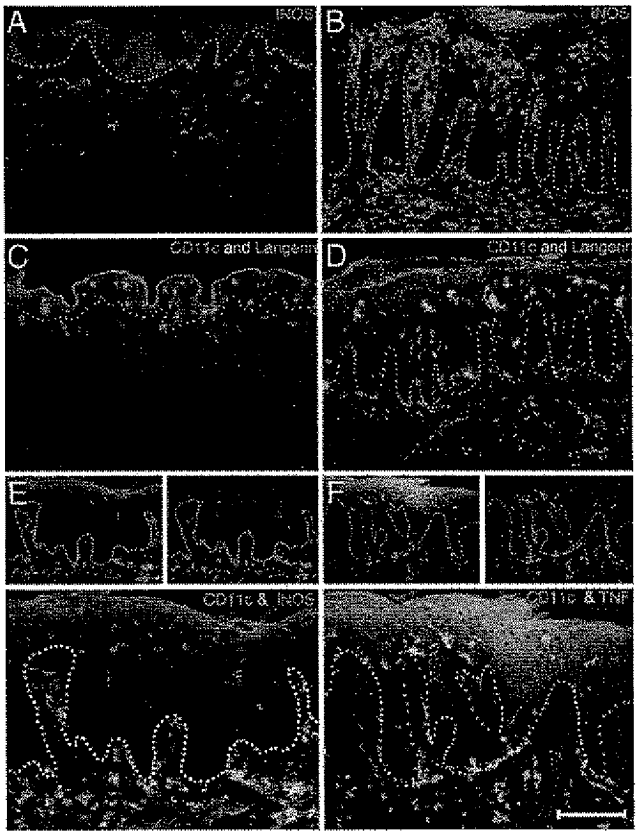

Fig. 1. iNOS and TNF double-positive CD11c$^+$ cells are greatly increased in psoriasis lesions. Single- and double-label immunofluorescence are shown. (Scale bar, 200 μm.) (*Insets*) Single color staining controls. (*A*) iNOS$^+$ cells in the dermis of normal skin. (*B*) iNOS$^+$ cells in the epidermis and dermis of psoriasis skin. (*C*) CD11c$^+$ cells in the dermis of normal skin. (*D*) CD11c$^+$ cells in the epidermis and dermis of psoriasis skin. No overlap with Langerin positive cells in the epidermis in *C* and *D*. (*E* and *F*) There is nearly complete overlap between CD11c and iNOS, and CD11c and TNF.

CD11c Identifies a Type of Dendritic Cell. We carried out additional two-color immunofluorescence studies to characterize the CD11c$^+$ cells. They were larger cells and all strongly HLA-DR positive (Fig. 2*A*). Some of the iNOS producers also expressed markers of maturing DCs, DC-LAMP and CD83 (Fig. 2 *B* and *C*), and TNF$^+$ cells were predominantly HLA-DR$^+$ (Fig. 2*D*). To confirm that CD11c was primarily marking DCs, we performed more sensitive flow-cytometric analyses of cells from psoriasis lesions. The large, CD11c$^+$ cells could be classified mainly as maturing DCs, because 95% were CD86$^+$, 86% were CD40$^+$, and 63% were CD83$^+$ (Fig. 2*F*). Only a small percentage of CD11c$^+$ cells were CD14$^+$ (12%), and these cells were HLA-DR$^{mid\text{-}low}$ and probably best classified as monocytes. In contrast, the CD3$^+$ cells in the lesions were primarily found in the small cell fraction (R3), and these cells failed to express CD11c and CD83 (Fig. 2*G*). Because DCs mature when emigrating from

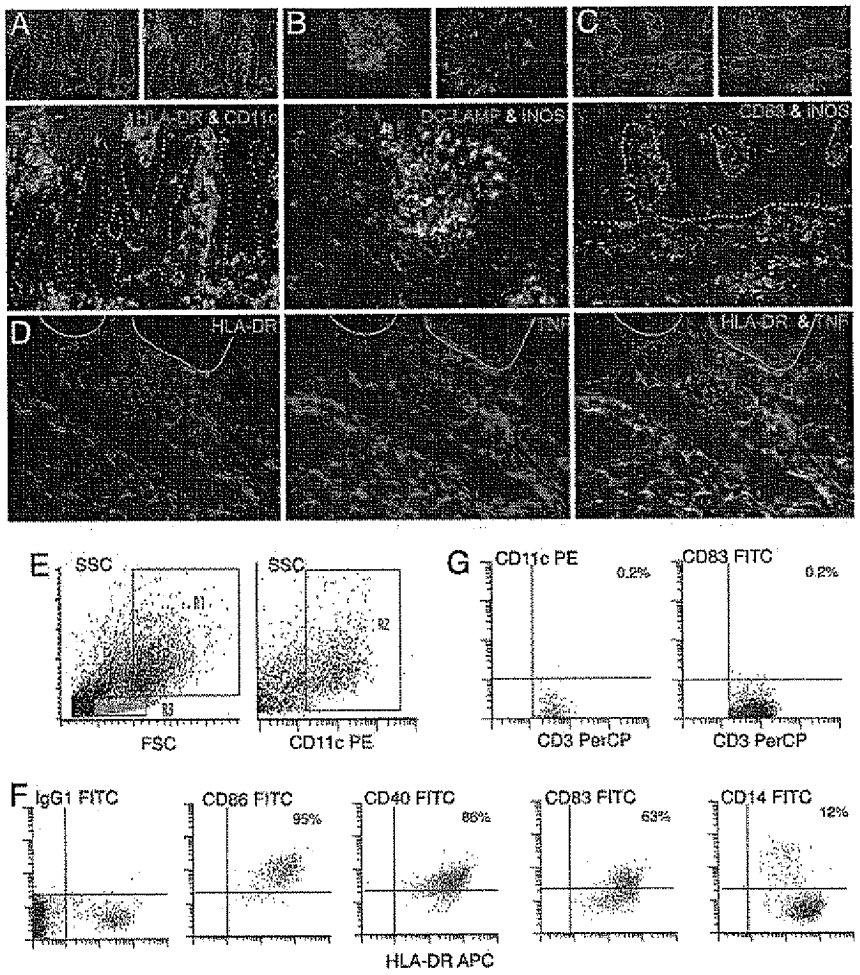

Fig. 2. CD11c identifies a type of DC. (*A–D*) Double-label immunofluorescence. (*Insets*) Single-color staining controls. (*A*) CD11c$^+$ cells are all HLA-DR$^+$. (*B* and *C*) Some iNOS$^+$ cells coproduce DC-LAMP and CD83. DC-LAMP and iNOS$^+$ cells are mature dermal DCs. (*D*) TNF$^+$ cells are predominantly HLA-DR$^+$. (*E–G*) FACS of dermal emigrants from a psoriatic patient. (*E*) R1, cells gated based on large cell size (FSC) and high side scatter (SCC); R2, CD11c-positive cells. (*F*) HLA-DR versus IgG1, CD86, CD40, CD83 and CD14 expression (R1+R2). Percent double-positive cells is shown in the upper right quadrant. (*G*) A lymphocyte gate (R3) indicates CD3-positive cells that have low CD11c and CD83 staining.

skin *in vitro*, the FACS experiments are likely to indicate that a high fraction of mature CD11c$^+$ DCs are in the emigrating population. Ongoing functional studies indicate that DCs emigrating from the dermis of psoriatic skin are strongly stimulatory for proliferative responses in autologous blood T cells, a functional property of DCs (25).

CD11c$^+$ DCs Are the Major Type of Leukocyte in Psoriatic Skin. Different types of leukocytes were then quantified by immunohistochemical staining, including the mean number of cells/low power field expressing CD1a, CD11c, CD83, CD3, and CD8. We compared normal human skin and psoriatic lesions before and during efalizumab therapy (anti-CD11a) (Fig. 3) (5). Perhaps the most surprising alterations in the leukocytes of psoriatic skin lesions were the CD11c$^+$ and CD83$^+$ DCs (Fig. 3 *B* and *C*). Normal human skin or uninvolved psoriatic skin contained similar numbers of CD11c$^+$ DCs (83 and 91 cells per field, respectively), which were exclusively localized in the dermis. In contrast in psoriasis, there was a large increase in CD11c$^+$ cells (411 cells per field, $P < 10^{-10}$ compared to normal skin), and nearly half (44%) of these CD11c$^+$ cells were found in the epidermis, mainly in the lower portions and along the dermo-epidermal junction. CD83$^+$ DCs were infrequent in normal skin (zero to five CD83$^+$ cells per field in normal skin), but increased to 108 CD83$^+$ DCs in active psoriasis lesions ($P < 10^{-5}$). The overall number of CD11c$^+$ cells in psoriatic skin actually exceeded the number of CD3$^+$ T cells.

CD11c$^+$ DCs Are Significantly Decreased After Efalizumab (Anti-CD11a) Treatment. Efalizumab treatment had a major impact on skin infiltration by CD11c$^+$ and CD83$^+$ DC populations. Overall, a 41% mean reduction in CD11c$^+$ cells was measured in patients treated with efalizumab ($P < 10^{-6}$), whereas placebo treatment produced no significant reduction. The reductions observed in K16$^-$ (high responder) patients was even greater, averaging 68% for CD11c$^+$ DCs, and 89% when the final number of DCs was corrected for numbers present in nonlesional skin. CD83$^+$ DCs were impacted even more by efalizumab treatment, as a 90% reduction in mature DCs was observed in high responders, with a 98% reduction when corrected by nonlesional skin cell counts. There was a mean of 49 CD1a$^+$ (Langerhans) cells per field in normal skin, 183 lesional CD1a$^+$ cells per field, and no significant reduction in number of CD1a$^+$ cells after efalizumab treatment. These cells were evenly distributed throughout the epidermis of normal and uninvolved skin, often concentrated in the upper spinous layer in lesional psoriatic

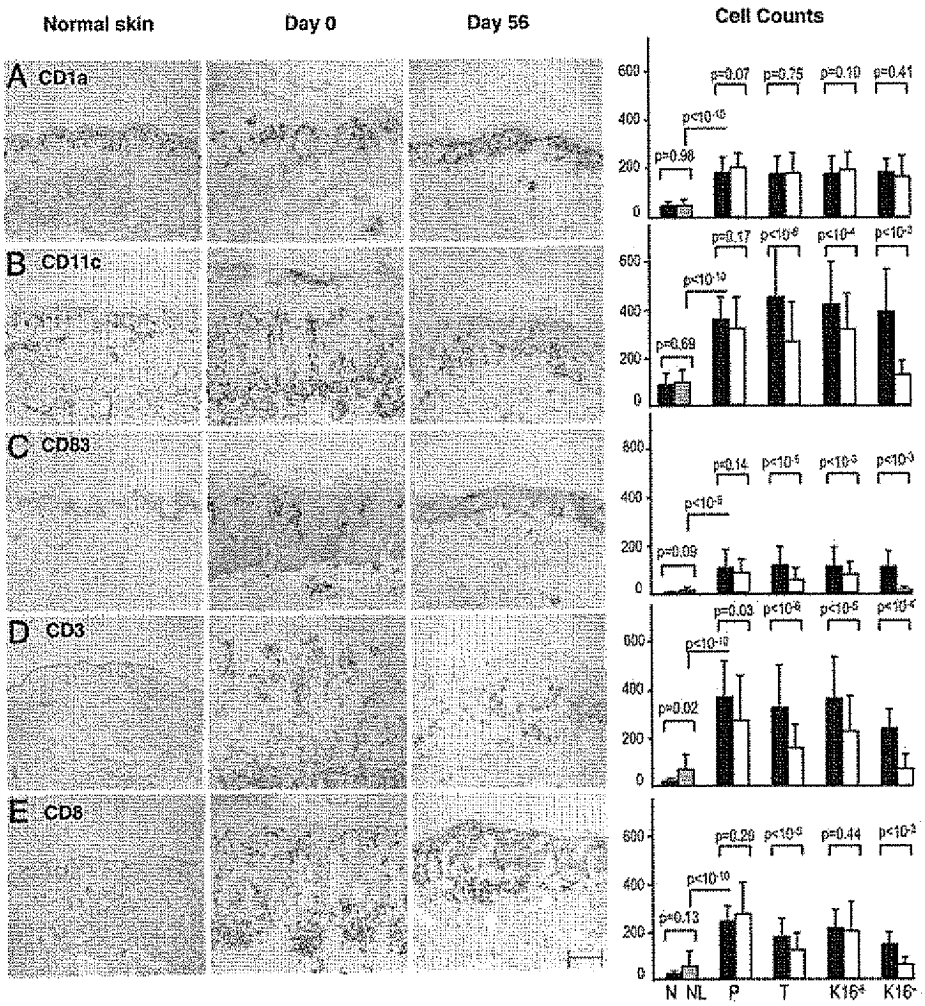

Fig. 3. CD11c$^+$ DCs are significantly decreased after efalizumab (anti-CD11a) treatment. Histomicrographs and cell counts of skin biopsies from a normal control and a treatment group patient (responder, K16 negative), on days 0 and 56. (Scale bar, 200 μm.) Mean number of total cells per low power field (10×) for CD1a (*A*), CD11c (*B*), CD83 (*C*), CD3 (*D*), and CD8 (*E*) (±SD) in normal skin (N, black), nonlesional psoriasis skin (NL, gray), and lesional skin of patients in placebo (P), treatment (T; all patients), K16$^+$ (non- and partial responders), and K16$^-$ (high responders) groups on days 0 (filled squares) and 56 (open squares). *P* values are shown for the indicated comparison.

epidermis, and restored to normal with therapy. Highly significant decreases in CD3$^+$ and CD8$^+$ cells were observed in efalizumab-treated patients (Fig. 3 *D* and *E*). Total CD3$^+$ cells declined by 47% in the patients who received efalizumab ($P < 10^{-6}$), and by 70% in K16$^-$ (high responders) ($P < 10^{-4}$). These results indicate that CD11c$^+$ cells, and not just T cells, are a major responsive element in successful CD11a-based therapy of psoriasis.

Changes in Number of CD11c$^+$ and CD83$^+$ Cells, as Well as iNOS mRNA Expression, Correlate Highly with Disease Improvement. We used efalizumab as a disease-modulating agent in these studies because it is a leukocyte-specific antibody, and both DCs and T cells express LFA-1. A recently developed statistical approach was used to compare disease improvement in individual patients to changes in specific leukocyte subsets (DCs and T cells) (22). The amount of psoriasis disease activity at the end of treatment was quantified as a "response score" (Fig. 4), a composite *U* score derived from measurement of epidermal thickness and K16 expression (positive or negative) on day 56. As shown in Fig. 4*A*, disease improvement (response score) could be related to changes in T cells or DCs. However, the CD11c$^+$ population showed the highest correlation with the response score ($r = 0.62$), whereas the correlation with T cell changes (CD3$^+$) was relatively poor ($r = 0.32$). Changes in CD83$^+$ DCs were also better correlated with response ($r = 0.52$) than T cells. We also examined the degree to which disease improvement during efalizumab treatment was related to altered expression of iNOS mRNA in skin lesions. As shown in Fig. 4*B*, response score was highly related to reduction in iNOS mRNA in individual patients ($r = 0.83$).

Therefore, we confirmed that myeloid (*ex vivo* peripheral blood monocyte-derived) DCs can synthesize high levels of iNOS mRNA by using real-time RT-PCR (Fig. 4*C*). Immature DCs were able to express iNOS, but the highest level of iNOS was measured in mature (CD83$^+$) DCs, which were differentiated *in vitro* by using a cytokine mixture (21). In comparison, resting T cells, activated T cells, blood monocytes, and a keratinocyte cell line (HaCaT cells) produced very little iNOS mRNA. We then assessed whether CD11c$^+$ cells might be reduced before clinical improvement. As shown in Fig. 4*D*, during the first 2 weeks of efalizumab treatment, there was a 50% reduction in lesional dermal CD11c$^+$ cells ($P = 0.006$), but only a 14% reduction in epidermal thickness (an indicator of clinical response). The early kinetics of reduction in CD11c$^+$ cells show that these cells are decreased before thinning of the epidermis and clinical improvement occurs.

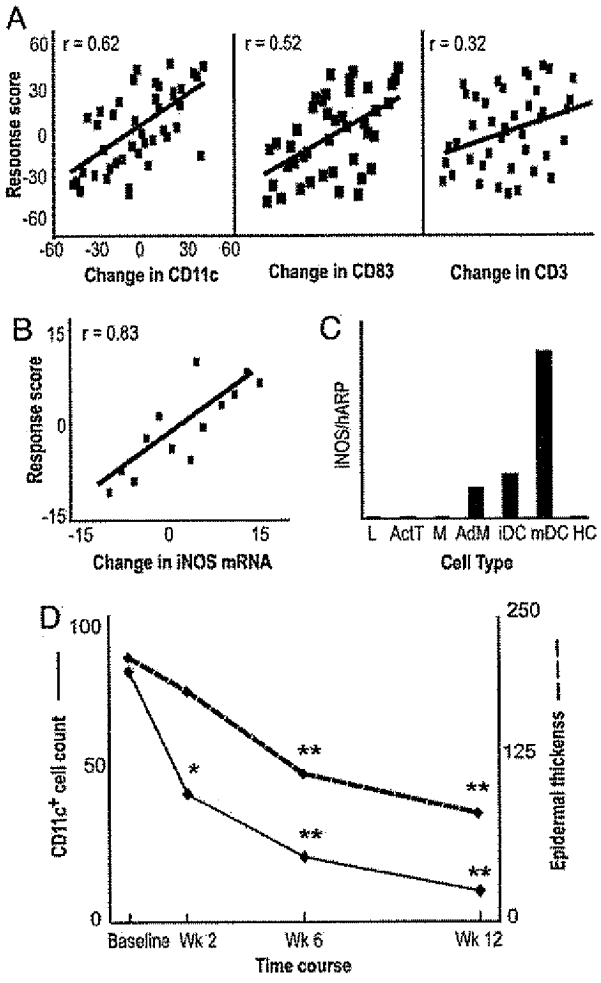

Fig. 4. Changes in number of CD11c and CD83 positive cells, as well as iNOS mRNA expression, correlate highly with disease improvement. (*A*) Correlation between response score and changes in DCs ($CD11c^+$ and $CD83^+$) and T cells ($CD3^+$) (*u* score) between day 0 and 56 for patients in the efalizumab-treated group. Correlation coefficients (*r*) are shown. (*B*) Correlation between response score and change in iNOS mRNA in lesional tissue between day 0 and 56 ($r = 0.83$). (*C*) *In vitro* monocyte-derived DCs produce iNOS mRNA. iNOS/HARP mRNA was measured by RT-PCR in lymphocytes (L), T cells activated by CD3/CD28 (ActT), monocytes (M), adherent monocytes (AdM), immature DCs (iDC), mature dendritic cells (mDC), and HaCaT cells (HC). (*D*) Dermal $CD11c^+$ cell counts/low power field (straight black line) and epidermal thickness (dotted black line) during therapy. During the first 2 weeks of treatment, there is 50% reduction in dermal $CD11c^+$ cells (*, $P = 0.006$) and a 14% reduction in epidermal thickness (not significant). **, $CD11c^+$ cell counts and epidermal thickness at weeks 6 and 12 compared to baseline, $P < 0.0001$.

Discussion

Two important mediators of inflammation, iNOS and TNF, are known to participate in the lesions of psoriasis (23, 26), and TNF blockade is an emerging therapy for this disease (15, 16). We now find that the iNOS enzyme and the TNF cytokine are abundant in a $CD11c^+$ cell in lesional dermis and epidermis. These $CD11c^+$ cells are the principal reservoir for these two mediators of inflammation and actually outnumber T cells in psoriatic lesions.

Our tissue and FACS staining data suggest that the abundant $CD11c^+$ iNOS- and TNF-producing cells in psoriasis are DCs, and similar to the Tip-DCs seen in mice (12–14), except that expression of CD11b is lacking on human cells (unpublished data). Murine Tip-DCs were defined as splenic $CD11c^+$, $CD11b^+$, $MHC\text{-}II^+$, $CD40^+$, and $CD86^+$ cells producing iNOS and TNF. Tip-DCs appeared to originate from circulating cells expressing CCR2 (receptor for monocyte chemotactic protein-1). They were largely absent in normal spleens, but accumulated in response to *Listeria* infection. Mice unable to recruit splenic $CCR2^+$ cells were severely compromised in their capacity to fight *Listeria* infection. The differentiation of Tip-DCs required MyD88, a central regulatory molecule in myeloid development activated by Toll-like receptors that is responsible for the induction of a variety of proinflammatory cytokines (27). Tip-DCs may be derived from monocytes, because cultured monocyte-derived DCs express iNOS (Fig. 4*C*) and TNF (unpublished data).

Previously, epidermal DCs in inflammatory skin lesions (atopic dermatitis and psoriasis) have been classified as Langerhans cells, inflammatory dendritic epidermal cells (IDECs), and plasmacytoid DCs, by FACS techniques (28–30). Tip-DCs might contain the IDEC population because both express the integrin CD11c (unlike Langerhans cells). However, IDECs express CD1a, suggesting that they might be a subset of activated Langerhans cells, and we were unable to detect CD1a or Langerin/CD207 expression on epidermal $CD11c^+$ DCs. In previous work, dermal DCs in psoriasis were identified by expression of factor XIIIa (31). We are finding an increase in $XIIIa^+$ cells in psoriasis lesions, but these cells are confined to the dermis, even in active psoriasis lesions, and are distinct from the Tip-DCs.

Efalizumab clearly blocks trafficking of $LFA\text{-}1^+$ T cells into psoriasis skin lesions (19). The observed DC reduction with efalizumab therapy may be due to either a direct effect on DCs (which express CD11a; ref. 32) or a direct effect on T cells, impacting on the DC–T cell relationship. We suspect that the DCs are a principal target because of our observation that the decrease in $CD11c^+$ DC numbers during therapy is faster than the reduction in epidermal thickness. Likewise, both T cells and $CD11c^+$ DCs are decreased in psoriasis lesions treated with other immunosuppressive therapies such as CTLA4Ig or alefacept (4, 6). Further proof that TNF- and iNOS-producing DCs could be direct inflammatory effector cells in autoimmune disease awaits the development of more DC-specific antagonists that can be used in clinical studies.

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1. Krueger, J. G. (2002) *J. Am. Acad. Dermatol.* **46,** 1–23.
2. Lew, W., Bowcock, A. M. & Krueger, J. G. (2004) *Trends Immunol.* **25,** 295–305.
3. Nickoloff, B. J. & Nestle, F. O. (2004) *J. Clin. Invest.* **113,** 1664–1675.
4. Abrams, J. R., Kelley, S. L., Hayes, E., Kikuchi, T., Brown, M. J., Kang, S., Lebwohl, M. G., Guzzo, C. A., Jegasothy, B. V., Linsley, P. S. & Krueger, J. G. (2000) *J. Exp. Med.* **192,** 681–694.
5. Papp, K., Bissonnette, R., Krueger, J. G., Carey, W., Gratton, D., Gulliver, W. P., Lui, H., Lynde, C. W., Magee, A., Minier, D., *et al.* (2001) *J. Am. Acad. Dermatol.* **45,** 665–674.
6. Chamian, F., Lowes, M. A., Lin, S. L., Lee, E., Kikuchi, T., Gilleaudeau, P., Sullivan-Whalen, M., Cardinale, I., Khatcherian, A., Novitskaya, I., *et al.* (2005) *Proc. Natl. Acad. Sci. USA* **102,** 2075–2080.
7. Lee, E., Trepicchio, W. L., Oestreicher, J. L., Pittman, D., Wang, F., Chamian, F., Dhodapkar, M. & Krueger, J. G. (2004) *J. Exp. Med.* **199,** 125–130.
8. Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., *et al.* (2003) *Nature* **421,** 744–748.
9. Murphy, C. A., Langrish, C. L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R. A., Sedgwick, J. D. & Cua, D. J. (2003) *J. Exp. Med.* **198,** 1951–1957.
10. Liu, Y. J. (2001) *Cell* **106,** 259–262.
11. MacDonald, K. P., Munster, D. J., Clark, G. J., Dzionek, A., Schmitz, J. & Hart, D. N. (2002) *Blood* **100,** 4512–4520.
12. Serbina, N. V., Salazar-Mather, T. P., Biron, C. A., Kuziel, W. A. & Pamer, E. G. (2003) *Immunity* **19,** 59–70.

13. Serbina, N. V., Kuziel, W., Flavell, R., Akira, S., Rollins, B. & Pamer, E. G. (2003) *Immunity* **19,** 891–901.
14. Tam, M. A. & Wick, M. J. (2004) *Trends Immunol.* **25,** 335–339.
15. Gottlieb, A. B., Masud, S., Ramamurthi, R., SAbdulghani, A., Romano, P., Chaudhari, U., Dooley, L., Fasanmade, A. A. & Wagner, C. L. (2003) *J. Am. Acad. Dermatol.* **48,** 68–75.
16. Leonardi, C. L., Powers, J. L., Matheson, R. T., Goffe, B. S., Zitnik, R., Wang, A. & Gottlieb, A. B. (2003) *N. Engl. J. Med.* **349,** 2014–2022.
17. Vallat, V. P., Gilleaudeau, P., Battat, L., Wolfe, J., Nabeya, R., Heftler, N., Hodak, E., Gottlieb, A. B. & Krueger, J. G. (1994) *J. Exp. Med.* **180,** 283–296.
18. Ferenczi, K., Burack, L., Pope, M., Krueger, J. G. & Austin, L. M. (2000) *J. Autoimmun.* **14,** 63–78.
19. Vugmeyster, Y., Kikuchi, T., Lowes, M. A., Howell, K., Chamian, F., Kagen, M. H., Gilleaudeau, P., Lee, E., Dummer, W., Pippig, S., *et al.* (2004) *Clin. Immunol.* **113,** 38–46.
20. Pope, M., Betjes, M. G., Hirmand, H., Hoffman, L. & Steinman, R. M. (1995) *J. Invest. Dermatol.* **104,** 11–17.
21. Lee, A. W., Truong, T., Bickham, K., Fonteneau, J. F., Larsson, M., Da Silva, I., Somersan, S., Thomas, E. K. & Bhardwaj, N. (2002) *Vaccine* **20,** Suppl. 4, A8–A22.
22. Wittkowski, K. M., Lee, E., Nussbaum, R., Chamian, F. N. & Krueger, J. G. (2004) *Stat. Med.* **23,** 1579–1592.
23. Ormerod, A. D., Weller, R., Copeland, P., Benjamin, N., Ralston, S. H., Grabowksi, P. & Herriot, R. (1998) *Arch. Dermatol. Res.* **290,** 3–8.
24. Nathan, C. & Shiloh, M. U. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 8841–8848.
25. Steinman, R. M. & Nussenzweig, M. C. (1980) *Immunol. Rev.* **53,** 127–147.
26. Feldmann, M. & Maini, R. N. (2003) *Nat. Med.* **9,** 1245–1250.
27. Janssens, S. & Beyaert, R. (2002) *Trends Biochem. Sci.* **27,** 474–482.
28. Wollenberg, A., Kraft, S., Hanau, D. & Bieber, T. (1996) *J. Invest. Dermatol.* **106,** 446–453.
29. Wollenberg, A., Wagner, M., Gunther, S., Towarowski, A., Tuma, E., Moderer, M., Rothenfusser, S., Wetzel, S., Endres, S. & Hartmann, G. (2002) *J. Invest. Dermatol.* **119,** 1096–1102.
30. Schuller, E., Teichmann, B., Haberstok, J., Moderer, M., Bieber, T. & Wollenberg, A. (2001) *Arch. Dermatol. Res.* **293,** 448–454.
31. Nestle, F. O., Turka, L. A. & Nickoloff, B. J. (1994) *J. Clin. Invest.* **94,** 202–209.
32. Nguyen, V. A., Ebner, S., Furhapter, C., Romani, N., Kolle, D., Fritsch, P. & Sepp, N. (2002) *Eur. J. Immunol.* **32,** 3638–3650.